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Parental effects of social density on mating behaviour, reproductive success, and longevity

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Parental effects of social density on mating behaviour, reproductive success, and longevity

by

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Author's Declaration

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Shaun Edward Rowlands

Parental effects of social density on mating behaviour, reproductive success, and longevity

Abstract

Organisms can adapt to changing environments, changing their investment strategies to increase their lifetime reproductive success. Contemporary evolutionary theory would suggest that organisms have evolved mechanisms which allow them to assess competition and acquire phenotypic responses that accurately responds to the environment, allowing for rapid changes in response to the social context. Selection would favour organisms which can make non-genetic parental effects, with parents evolving an ability pass on plasticity responses that optimize offspring fitness related traits to suit ecological challenges. In this thesis , I investigate how the social density affects the mating behaviour and offspring production of the parent and whether they use their current social environment to assess the reproductive opportunities and competition their offspring are likely to encounter. To determine the impact social density has on mating behaviour and whether there is a parental effect being conferred to the offspring I will raise two generations of male and female D. melanogaster in different social density treatments and measure their mating behaviours and how many offspring they produce.

Female *D. melanogaster* raised at high density produce fewer offspring, but of a higher quality, which are larger and have higher disease resistance. Females which have been kept in low density mixed-sex environments are less likely to

reject the males mating attempt and will produce more eggs than high density female. Given these responses, I predicted that the females raised at high density would confer mating behaviour traits to their offspring to give them an advantage in the social environments they were expected face. Here the results of my study suggest that there is a parental effect on mating behaviour being conferred on to the offspring in response to the mother's density, with high density mothers producing daughters which are more attractive to the male and which mate for longer (Chapter 2).

I then used tested whether male *D. melanogaster* could pass on parental effects to their offspring in response to their social environment (Chapter 3). *D. melanogaster* males that anticipate a higher risk of sperm competition take longer to initiate courtship, have a longer mating latency and lengthen their mating time. The results of this study suggest that the male flies which anticipate higher levels of sperm competition, delay mating with the female. This suggests that the male, anticipating fewer mating opportunities due to the competitive environment has a higher threshold that the female must pass, than the males that do not anticipate high levels of sperm competition. The low-density males, which courted faster than the males raised at high density, may try, and capitalise on the mating opportunity, as more mating opportunities are expected. The results of this study suggest that we can use male, or sperm, competition to encourage males to initiate mating. Being able to reduce male choosiness may be a useful tool in conservation where access to females is limited, such as in *ex-situ* conservation programmes, although further studies would need to be conducted as to whether the results found *D. melanogaster* are conferred in other species.

This study showed that mating latency, which was used in this study as a proxy of male attractiveness, was not significantly affected by the social density the male was reared in. This suggests that keeping males at high density social groups does not impact that attractiveness to the female, which suggests that keeping males in a high social group can increase their willingness to mate with a presented female without affecting the female's attraction to the with the male. In fact, this study shows that males who experience no rival male competition prior to mating were more likely to be rejected by the female. That the number of offspring produced were also unaffected by the male's social environment suggests that if a breeding programme were to select a male that was raised at high density with other males then they would be more likely to choose to court the female, less likely to be rejected by the female and they would suffer no loss in quantity of offspring produced.

This study also tested the impact the paternal social density would have on their son's mating behaviour. Studies on *D. melanogaster* suggest that parental density treatment has significant intergeneration effects on both juvenile and adult fitness. The results of this study show that whilst the sires adapted their mating behaviour in response to the social density they experienced, these responses were not passed down to their sons. These results suggest that sons mating behaviours are unaffected by the social environment of their fathers. This would be advantageous to *in-situ* and *ex-situ* breeding programmes, as it allows males to be placed in high social environments, when necessary, without there being a negative impact on their son's mating behaviour. This model may only be suitable for species which cultivate in ephemeral resource matches, such as *D. melanogaster*, where crowding in transiently available rich patches is a key component of their natural ecology. In Chapter 1, I discuss how crowding affects

mating behaviours and success in mammals, where it can have a negative affect on mating success of quality of offspring produced. This study builds on the evidence that the parental social density has an impact on mating behaviour and success and suggests that future studies investigate how well *D. melanogaster* works as a model across species.

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Chapter 1: General Introduction

The environment in which an organism develops can have a significant impact on their physiology, morphology, behaviour and fitness. There is a growing body of evidence suggesting that the mechanisms and genetic variations which affect fitness should not be heritable, but instead allow organisms to adapt their offspring production in accordance with their own health and their environment (Falcooner, 1960; Partridge, 1980; McCollough, 1999; Crocker and Hunter, 2018). Contemporary evolutionary theory (Hendry and Kinnison, 1999) would suggest that organisms have evolved mechanisms which allow them to assess competition and acquire phenotypic responses that accurately responds to the environment, allowing for rapid changes in response to the social context (Kasumovic *et al.*, 2008; Allen *et al*., 2008; Adler and Bonduriansky, 2013; Bretman *et al*., 2013). Selection would favour organisms which can make non-genetic parental effects, with parents evolving an ability pass on plasticity responses that optimize offspring fitness related traits to suit ecological challenges (Bonduriansky and Day, 2009; Crean *et al*., 2013).

Some species adapt to high density environments by reducing the number of offspring produced (Badyaev, 2005; Crocker and Hunter, 2008; McCollough, 1999), or through plastic response, such as organ size or mating behaviours. (Stockley and Seale, 2001). When male dung flies, *Scatophaga stercoraria*, were raised at high density they responded by producing larger testes (relative to thorax size) Stockley and Seale, 2001). This suggests that males reared under high larval density conditions anticipating increased sperm competition raise their overall reproductive success by increasing investment to the testes (Linklater *et*

al., 2007). When the *S. stercorcaria* lava were raised at high density with limited resources they had an increased mortality rate at the end of the breeding season than the lava raised at low density (Blackenhorn, 1998), which could be used as a paradigm for species which cultivate in ephemeral resource matches, including the fruit fly, *Drosophila melanogaster*, where crowding in transiently available rich patches is a key component of their natural ecology (Dasgupta *et al.*, 2019).

In mammals it was found that animals became less fertile because of overcrowding, with animals being kept in large concentrations often having reduced birth rates (Wynne-Edwards, 1986; O'Malley *et al*., 2008). This has led some evolutionary biologists to suggest that the animals have a biological mechanism for producing the optimum number of offspring that can be produced to ensure that most survive to adulthood. This would prevent energy being wasted producing an excess amount of offspring that have little to no chance of making it to adulthood. In this theory the overcrowding foreshadows famine, when the female experiences overcrowding(through population size) her body adjusts accordingly (Dawkins, 2016). If the parent is adjusting offspring number in anticipation of famine and can pass on non-genetic parental effects, then they may have the ability to pass on traits which confer an advantage to their offspring in the harsh conditions.

Empirical studies on adaptive responses have tended to concentrate on whether parental effects are adaptive, with theoretical works focusing on the short-term responses of trait selection for the parental environment. It has been

demonstrated that parental effects can be a form of plasticity that spans generations (Uller, 2008). Parental effects can be adaptive, especially under fluctuating environments, with the development depending on the reliability of information provided by parents and that obtained within the individuals non-parental environment (Uller, 2008; Dasgupta *et al*.,2019). With limited resources selection might favour parental effects that reduce the number of offspring produced (Badyaev, 2005), selecting instead on producing offspring better adapted to survive to reproductive age and better at securing mates, thus increasing the offspring's individual fitness. For transgenerational phenotypic effects to optimize fitness the density, or resource, fluctuations must be predictable (Dasgupta *et al*., 2019).

Studies on *Drosophila melanogaster* suggest that parental density treatment has significant intergeneration effects on both juvenile and adult fitness (Dasgupta *et al.*, 2019). Males reared at high density (~200 per vial) have a paternal effect on juvenile fitness, with males from high density sires producing more offspring than males from low density fathers. The same study also found that males reared at "Intermediate density" (~150 per vial) produced smaller sons that were inferior at acquiring mates. This suggests that there is a density threshold for parental effects, although it is difficult to determine how as even the low groups in this study were relatively large (~150 per vial).

The results of the study tentatively point toward a non-genetic paternal effect as the cause behind observed effects of the treatment groups (Bonduriansky and Day, 2009; Dasgupta et al., 2019), however as the study investigated the intensity of selection on non-stable population, and therefore unpredictable. The number of offspring produced is one trait that may be passed on from the parent to the offspring, however, this study also wants to look at how whether social density effects other traits, such as mating behaviour.

Mating and courtship behaviour is energetically expensive and males that anticipate mating competition may also adjust their mating behaviour to reduce the risk of wasting energy investments on unsuccessful mating (Nurr, 1984; Harshman and Zara, 2007; Bretman *et al*., 2013). *D. melanogaster* males that anticipate increased male mating rivals (perceived sperm competition) will take longer to initiate courtship, with an increased latency between mating, suggesting that they take time to decide whether to hold for a better mating candidate, limiting the amount of energy wasted on mating behaviour with a lower quality female. (Bretman et al., 2013; Marie-Orleach et al., 2018).

Mating and courtship success is also influenced by female mate choice. This occurs wherever there is a bias towards certain male phenotypes (Maynard-Smith, 1987). In many species there is a selection bias toward choosy females, as males will vary in physical traits that affect the female's fitness (Andersson, 1994). Female choice theory suggests that the female must be attracted to the male to successfully mate. Males within the population will have different abilities

of attracting females, (e.g., courtship displays, body size, fighting ability, olfactory stimulation) with the more attractive males being able to increase their lifetime fitness through having more successful mating's than their less attractive rivals. Selection would favour these attractive males that can attract and impress best with the females, increasing their lifetime reproductive success (Andersson, 1994).

Mate choice, however, does involve a cost, with the female rejecting guaranteed opportunities of mating in favour of holding out for preferred mating conditions and must therefore allow the female to gain from mating with some males over others. The male may provide the female with direct benefits, elevating her fecundity, which would outweigh the costs of choice (Iwasa & Pomiankowski, 1999). Female mate choice can be either absolute, with the male needing to pass a threshold before mating is accepted, or relative, where the mating success chance is relative to the number and quality of other males available to the female at the time (Hoikkalla and Aspi, 1993; Lande 1981).

Drosophila species have complex courting behaviour, which include the male chasing the females, tapping the female with his forelegs, and the male producing a courtship song through the flexing of his wing (Villela and Hall, 2008), which enables the female to observe the male and assess his quality before deciding to mate or not. The female can accept to mate with the male, delay the decision to allow her to obtain more information about competing males or reject him as unsuitable (Hoikkalla and Aspi, 1993). Male *Drosophila* species that are exposed

to mating rivals take longer to court and mate, than those kept singularly, with an increased latency between mating (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018).

In this study, I investigate how the social density affects the mating behaviour and offspring production of the parent and whether they use their current social environment to assess the reproductive opportunities and competition their offspring are likely to encounter. To determine the impact social density has on mating behaviour and whether there is a parental effect being conferred to the offspring I will raise two generations of male and female D. melanogaster in different social density treatments and measure their mating behaviours and how many offspring they produce (Table 1), comparing the first generation and second generations, to measure the parental effect. I predict that the offspring's mating behaviour and offspring will be affected by the social density of their parent. If the offspring's mating behaviour is influenced by the parents' social density, then it would suggest that a non-genetic parental effect has been passed on to the offspring.

Table 1. Key words used in this paper, with definitions and whether they are determined by the male or female.

Term	Definition	Determined by:
Offspring produced	A count of how many offspring successfully eclosed to adulthood after a single mating of the parent.	
Courtship latency	The time taken from when the flies were first introduced into the vial to when the male began courtship, defined as the first wing flex.	Courtship latency is determined by the male, as they choose if they will initiate courtship and when.

Mating latency	The time taken from courtship beginning to the male mounting the female.	Mating latency is determined by the female, she chooses whether to accept or reject the male and when. Mating latency can also be affected by the male and how much effort he puts into courting.
Mating time	The time from when the male first mounts the female to when he detaches.	Mating time is determined by the male.
Longevity	The number of days the male is alive for, measured from the day they eclose from the pupae stage into adult flies.	

Chapter 2: Mating Behaviour Based on Maternal Environment

2.1 Introduction

The environment an organism develops in can influence their and their offspring's development. They can make plastic adjustments for the ecological challenges they or their offspring are likely to find themselves in (Bretman *et al.*, 2013; Kasumovic *et al.*, 2008). There are two different points in the life cycle that this epigenetic reprogramming can occur, during pre-fertilisation in the germ cells development and post fertilisation (Jirtle and Skinner, 2007).

It has been demonstrated that in mammals the mother can confer responses from her environment into the developing foetus. When the mother experiences stressful environments during earlier stages of pregnancy she can respond by producing more female offspring (Kremme *et al.*, 2015). Female offspring mature faster, which increases the likelihood of them reaching sexual maturity and reproducing within the stressful environment the mother anticipates them undergoing. When pregnant mothers experience malnourishment, she exposes the developing foetus to undernutrition. The foetus can respond, anticipating limited food availably, by developing mechanisms that would be advantageous during periods of limited food availably malnutrition, such as being more efficient in metabolising food energy and retaining weight (Beauchamp *et al.*, 2015).

The ability to confer responses from the mother's environment on to the offspring prior to fertilization has also been demonstrated in animals that do not develop the foetus or nurture the young, such as insects. There are examples of environment-dependent effects, such as competition, quality of resources, light and temperature, on mothers' offspring, which reflect the variation the maternal provisioning (Mousseau & Fox, 1998; Bonduriansky and Head, 2009; Zhan et al., 2010). The female can assess and transfer responses to environmental variation to offspring through maternal effects, such as egg provisioning, which in turn impact gene expressions, by turning specific genes on or off (Bretman et al., 2013; Arsenault et al., 2018). There is a trade-off between quality and quantity when producing offspring and mothers provisioning offspring balance the benefits of producing a few large, fitter offspring with the cost of decreased fecundity (Allen et al., 2008). When females are exposed to high density environments, resources are more limited, so they can struggle to allocate resources in maternal provisioning, which can lead to inferior quality progeny with reduced fitness (Christian and Lemunyan, 1958). Prasad et al. (2003) found female Drosophila melanogaster raised at high density produce less quantity of progeny but at a higher quality, with higher disease resistance (Mitchell and Read, 2005), by investing more resources in each of them, thereby giving them a better start for the impending conditions. Female guppies (Poecilia reticulata) reared in environments with limited food or prominent levels of competition produce larger offspring, priming the offspring for better competitive ability. (Reznick and Reznick, 1993; Bashey, 2006).

Maternal effects have been studied in a range of animals. For example, Allen *et al.* (2008) found that brown bryozoan, *Bugula neritina,* offspring size was adaptive and depended strongly on the intensity of intraspecific competition that offspring experience, and mothers would differentiate provision according to the environment their offspring would encounter. Offspring size had no effect on offspring performance in benign environments, with maternal fitness being increased by provisioning offspring to be numerous as opposed to large, whereas in intermediate densities with high competition selection favoured fewer but larger offspring (Allen *et al.*, 2008). Through manipulation of the fly, *Telostylinus angusticollis*, parental larval diet quality Bonduriansky and Head (2007) found that the maternal diet quality affect early life history, such as egg-size and development time, whereas paternal diet quality affected their progenies later in life, such as their adult body size. There is evidence from studies which show that events occurring early in life can have long-term effects on offspring phenotype (Curley *et al*., 2007). Evidence from human and other animal studies shows that aversive events occurring early in life can have long-term effects on offspring phenotype. Rhesus monkeys, *Macca mullatta*, reared without their mothers are behaviourally inhibited with increased stress responses and impairments in social and reproductive behaviour as adults (Ruppenthal, 1976).

Although relatively unexplored there have been studies on how the female social environment impacts the mating behaviour and offspring produced. Females which have been kept in low density mixed-sex environments are less likely to reject the males mating attempt than females from high density (Lehmann, 2007).

Suggesting that females which anticipate higher potential encounters are more discriminating when selecting mates. Not only are females which have been housed with other female more reluctant to mate they also have a longer mating latency with a shorter mating time than females kept in solitude (Churchill *et al*., 2021), which again suggest the females can anticipate the likelihood of a more suitable mate or female competitor coming and react accordingly. Females who have been exposed to female competition will lay fewer eggs (Churchill *et al*., 2021), therefore I would predict that the high-density females in this study will produce more adult offspring than the low-density females. There is a gap in the research for how the maternal social environment can affect mating behaviour and offspring production of her daughters, conferring environmental responses through the germline into the next generation. Most existing studies have focused on the effects of maternal rearing of the young. Females can pass on transgenerational responses to stressors in their environment. If a *D. melanogaster* female has experienced harassment from multiple males (without successful mating), she produces daughters with suboptimal fitness consequences (Zajitschek *et al*., 2018). Daughters from mothers who experienced high levels of sexual interaction produce more offspring, but at a cost to longevity and offspring survival (Dowling *et al*., 2014).

In this study I raise F0 female *D.melanogaster* in different densities and observe mating behaviours and offspring production from their F1 daughters raised in equal density. I predict that the mothers will be able to confer responses from their environment on to their offspring, therefore daughters from high density

mothers are predicted to have a longer mating latency, with a shorter time and to produce more offspring.

2.2 Methods

All flies used for experiments came from stock flies originating from a Canton-S stock population and were kept at 25°C, on a 12hour light: dark cycle. Stock populations are housed in 40ml plastic vials containing 7ml of an agar-based medium (40g of yeast and sucrose per litre), hereafter referred to as standard vials. Stock flies are raised in standard vials of approximately 25 *D.Melanogaster*, all vials are pooled and randomly distributed into new vials every ten days to minimise effects of inbreeding and drift.

Test flies were collected and sexed under ice anaesthesia from the stock vials within six hours of eclosion to ensure virginity and transferred to one of two treatments: solitary (1 female per vial), or groups (3 females per vial), where they were kept for seven days.

At seven days old, females were translocated to a standard vial for mating with a seven-day old virgin male which will have previously been housed in a grouped vial (3 males per vial) since eclosion. Mating behaviours were observed live, and courtship latency, mating latency and mating time, recorded in seconds. Females that do not mate within 30 minutes of being introduced were excluded from the trial and expunged along with all the males who did not successfully mate.

The standard vials containing successfully mated females were placed back in the incubator, the females are left to oviposit eggs for 22-24 hours, before being expunged. The vials were left in the incubator and after 21 days the emerged adults were counted, and three virgin females were taken from each vial and placed in a standard vial together, forming sister groups.

These daughter flies were kept in their vials for seven days before being translocated into a standard vial with a seven-day old virgin male which has previously been housed in a grouped vial (3 males per vial) since eclosion. The mating behaviours were observed live, and courtship latency, mating latency, and mating time recorded. After successful mating had been observed the male flies and unsuccessfully mated females are expunged and the mated females left in the vials to oviposit for 22-24 hours before being expunged themselves. The vials will be incubated at 25^{o}C for 21 days, where after the emerged adults are counted.

Data analysis and statistics

There were 21 F0 Flies used in this experiment, 11 high-density and 10 low-density. Once the flies that were not courted with or rejected the courting male were removed there were 9 high-density and 8 low-density F0 females. From these 17 F0 females the F1 generation was collected, again the flies that were not courted or rejected the courting male were removed leaving a final sample size of 49 flies, 28 from high-density parents and 21 from low-density parents.

The data was analysed using R v3.6.3 (R Core Team, 2014), and the R packages “dplyr”, “ggplot2”, “tidyr” and “lme4”. The effects of density treatment on F0 mating behaviour were analysed using a linear mixed effect model used the mating behaviour as the dependent variable and the density as independent variable (fixed effect). The F0 rearing vial was included in the model as a random effect to account for the rearing vial environment, as there were 4 groups of 3 flies sharing a vial.

A Shapiro-Wilk test was also used to test F0 generation residuals for normality, the results showed the courtship latency did have significant departure from normality ($W = 0.91$, $p = 0.002$), mating latency did have significant departure from normality ($W = 0.93$, $p < 0.006$), mating time did have significant departure from normality ($W = 0.93$, $p = 0.004$), and offspring number did not have significant departure from normality ($W= 0.96$, $p = 0.15$). I was unable to get the courtship latency, mating latency, or mating time normally distributed, even using log-transformation, however I decided to use them in the mixed model anyway as it was more important to be able to include the random effects than to fulfil the assumption of normality.

A Shapiro-Wilk test was also used to test F1 generation residuals for normality, the results showed the F1 courtship latency did not have significant departure from normality ($W = 0.96$, $p = 0.12$), F1 mating latency did not have significant departure from normality ($W = 0.96$, $p < 0.12$), F1 mating time did not have

significant departure from normality (W = 0.97, p = 0. 0.36), and F1 offspring number did not have significant departure from normality (W= 0.98, p = 0.42).

To test the effect, the F0 generations' social density had on their mating behaviour and offspring produced I used a linear model with the mating latency as the dependent variable and the social density as a fixed effect. Vial and dam were included as random effects to account for the vial of the dam and because three daughters of the same dam were housed together in groups.

2.3 Results

Density Effects on Mating Behaviour

Not all F0 females were courted (low density: 80% (8 out of 10); high density: 91% (10 of 11)). The density the female was kept in did not have a significant effect on the likelihood of being courted or successfully mating ($X^2 = 4.54$, d.f. = 20, p = 0.9).

Courtship Latency in Response to Social Density

Females from a high density were courted significantly faster than females from a low density ($F_{1,10} = 7.19$, $p = 0.02$; Figure 1a).

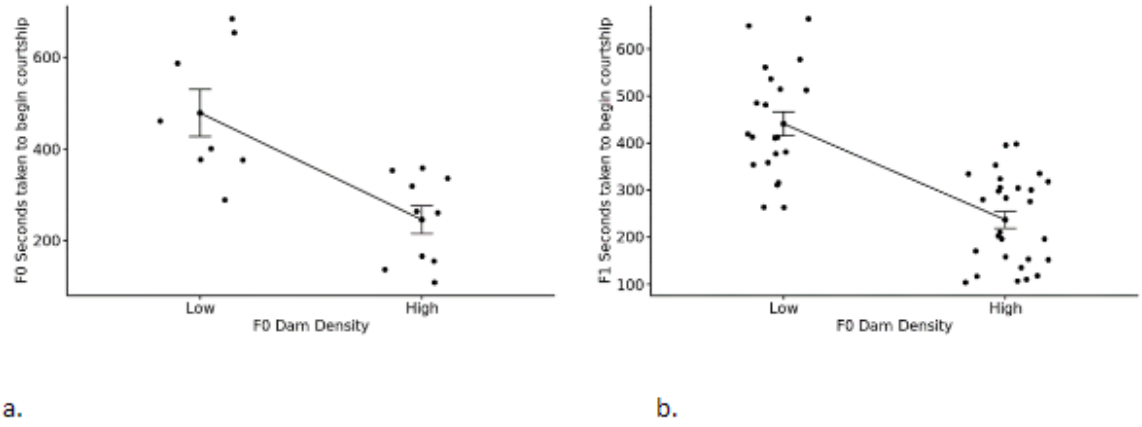


Figure 1. The effect the dam's social density had on courtship latency.
a. How long the male took to begin courting the mother (y-axis; in seconds), as a response to her social density (x-axis). The females kept at high density were courted significantly faster than the females kept at low density.
b. How long the male took to begin courting the daughter (y-axis; in seconds), in response to her mother's social density (x-axis). Daughters of high-density mothers were courted with significantly faster than females from low density mothers.

Dams were split into two density groups, low density (1 fly per vial) and high density (3 flies per vial). All daughter flies were kept at the same density (3 flies per vial). The females kept at high density were courted significantly faster than the females kept at low density.
Bar shows standard error.

As with the F0 generation, not all F1 female flies were courted, 88% (21 of 24) of females from low density mother's and 93% (28 of 30) of females from high density mothers and all, except for one daughter from a high-density mother, that were courted with mated and produced offspring. The density the mother was kept in did not have a significant effect on the likelihood of the daughter being courted (X^2 = 11.83, d.f. = 53, p = 1.0) or successfully mating (X^2 = 10.28 d.f. = 49, p = 1.0)

Female flies from a high-density environment had a significantly shorter courtship latency than female flies from a low-density environment ($F_{1,8.4}$ = 14.31, p = 0.005; Figure 1b).

Male flies did not spend longer courting females from low density environments than they did courting flies from high density environments ($F_{1,16}$= 0.04, p = 0.85; figure 2a).

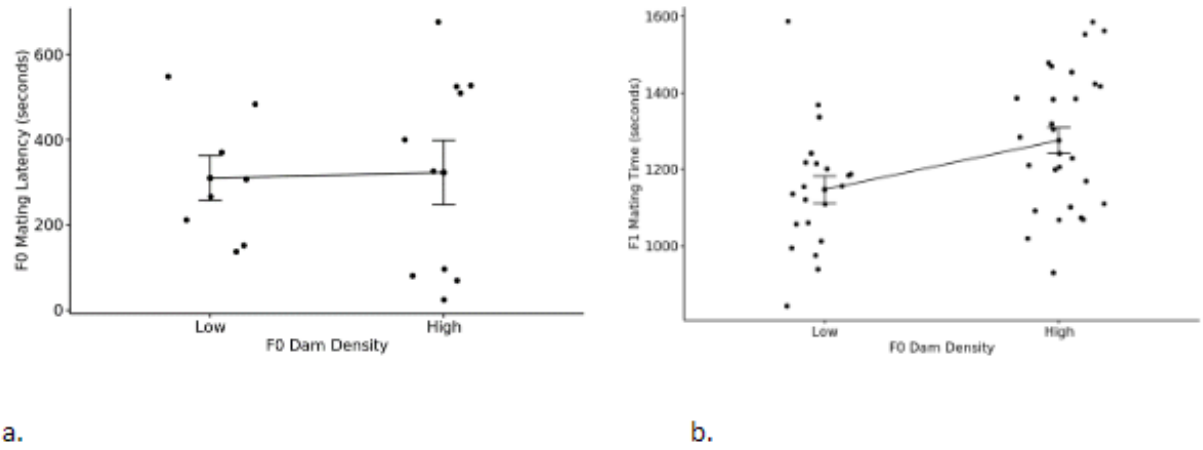


Figure 2. The effect the dam's social density had on mating latency.

a. The length of time (seconds) it took between the male beginning his courtship ritual and the female allowing him to mount. X-axis shows the F0 females' social density and y-axis shows mating latency, Bar shows standard error. Male flies did not spend longer courting females from low density environments than they did courting flies from high density environments.

b. The length of time it took for the mating male to mount the F1 female after initiating courtship. X-axis shows the mothers (F0) social density to test whether the mother's social density affected daughters mating latency. Bar shows standard error. Daughters from low density mothers did not have a longer mating latency than daughters from high density mothers.

Dams were split into two density groups, low density (1 fly per vial) and high density (3 flies per vial). All daughter flies were kept at the same density (3 flies per vial). The females kept at high density were courted significantly faster than the females kept at low density.

Daughters from low density mothers did not have a longer mating latency than daughters from high density mothers (F1,16.4= -1.79, p = -0.22; figure 2b)

Mating Time in Response to Social Density

Female flies from low density social environments did not have a shorter mating time than females from a low-density environment ($F_{1,16}$= 0.24, p = 0.63; figure 3a)

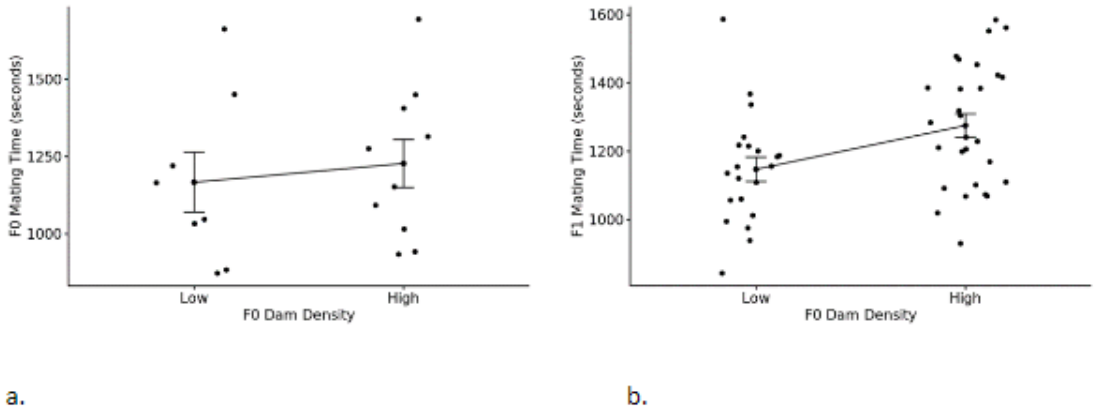


Figure 3. The effect the dam's social density had on mating time.

a. The amount of time (in seconds) the mating male spend latched on to the F0 female (y-axis). The x-axis shows the Dams social density group, testing the impact the social environment had on mating time. Bar shows standard error. Female flies from low density social environments did not have a shorter mating time than females from a low-density environment.

b. The amount of time (in seconds) the mating male spend latched on to the F1 daughter fly (y-axis). The x-axis shows the FO Dams social density group, testing the impact the mother's social environment had on daughters mating time. Bar shows standard error. Daughters from low density mothers did have a shorter mating time than daughters from high density mothers.

Dams were split into two density groups, low density (1 fly per vial) and high density (3 flies per vial). All daughter flies were kept at the same density (3 flies per vial). The females kept at high density were courted significantly faster than the females kept at low density.

Daughters from low density mothers did have a shorter mating time than daughters from high density mothers (F1,15.8= 4.56, p <0.05; figure 3b).

Offspring Produced in Response to Social Density

Female flies that were raised in a high-density environment did not produce more offspring than female flies that were raised in a low-density environment ($F_{1,2.8}$= 9.45, p = 0.06; figure 4a).

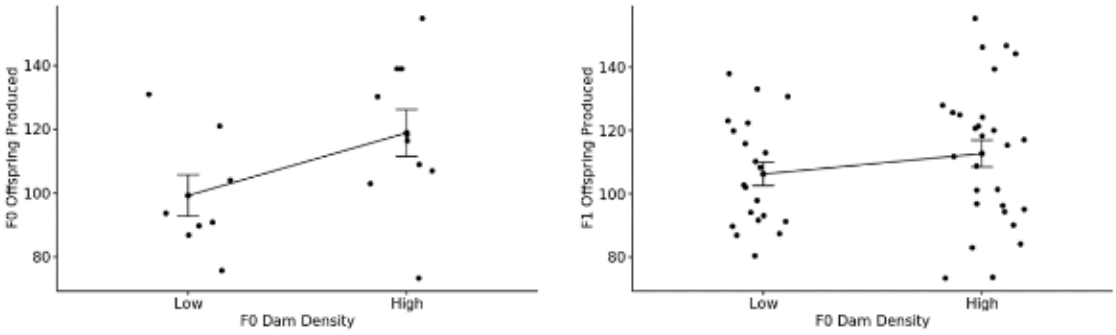


Figure 4. The effect the dam's social density had on offspring produced

a. The number of offspring that survived until adulthood (y-axis) following a single mating from the F0 female fly. The x-axis shows the F0 Dams social density to test whether the social environment impacted the fly's offspring production. Bar shows standard error. Female flies that were raised in a high-density environment did not produce more offspring than female flies that were raised in a low-density environment.

b. The number of offspring that survived until adulthood (y-axis) following a single mating from the F1 daughter fly. The x-axis shows the F0 Dams social density to test whether the mother's social environment impacted the daughters offspring production. Bar shows standard error. Daughters from low density mothers produced no more offspring than daughters from high density mothers.

Dams were split into two density groups, low density (1 fly per vial) and high density (3 flies per vial). All daughter flies were kept at the same density (3 flies per vial). The females kept at high density were courted significantly faster than the females kept at low density.

Daughters from low density mothers produced no more offspring than daughters from high density mothers ($F_{1,47}$= 4.56, p = 0.28; figure 4b).

Courtship Latency Correlation test

There is a significant positive correlation between the mother's courtship latency and her daughter's courtship latency (R(47) = 0.82, p = <0.001; Figure 5) .

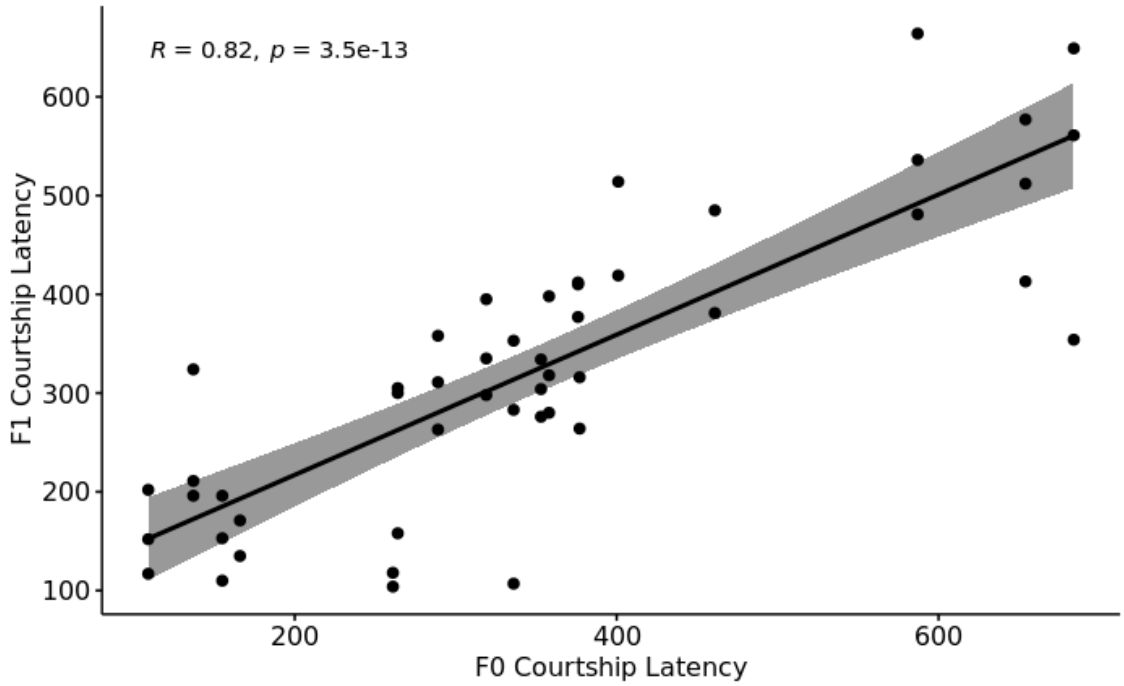


Figure 5. The correlation between the Dams (F0) courtship latency and the daughters (F1) courtship latency. Black line shows line of best fit. grey shadowing shows the accompanying 95% confidence interval.

Mating Latency Correlation test

There is a significant positive correlation between the mother's mating latency and her daughter's mating latency (R(47) = 0.43, p = 0.002; Figure 6.

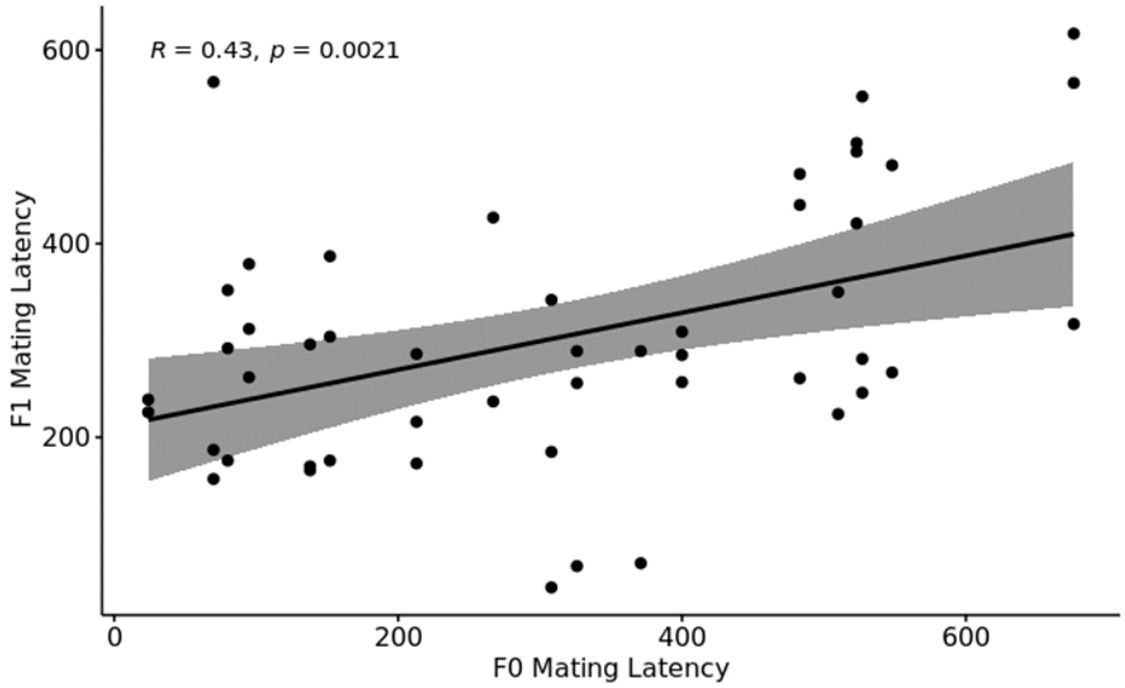


Figure 6. The correlation between the Dams (F0) mating latency and the daughters (F1) mating latency. Black line shows line of best fit. grey shadowing shows the accompanying 95% confidence interval.

Mating Time Correlation test

There is a significant correlation between the mothers mating time and her daughters mating time (R(47) = 0.70, p = <0.001; Figure 7).

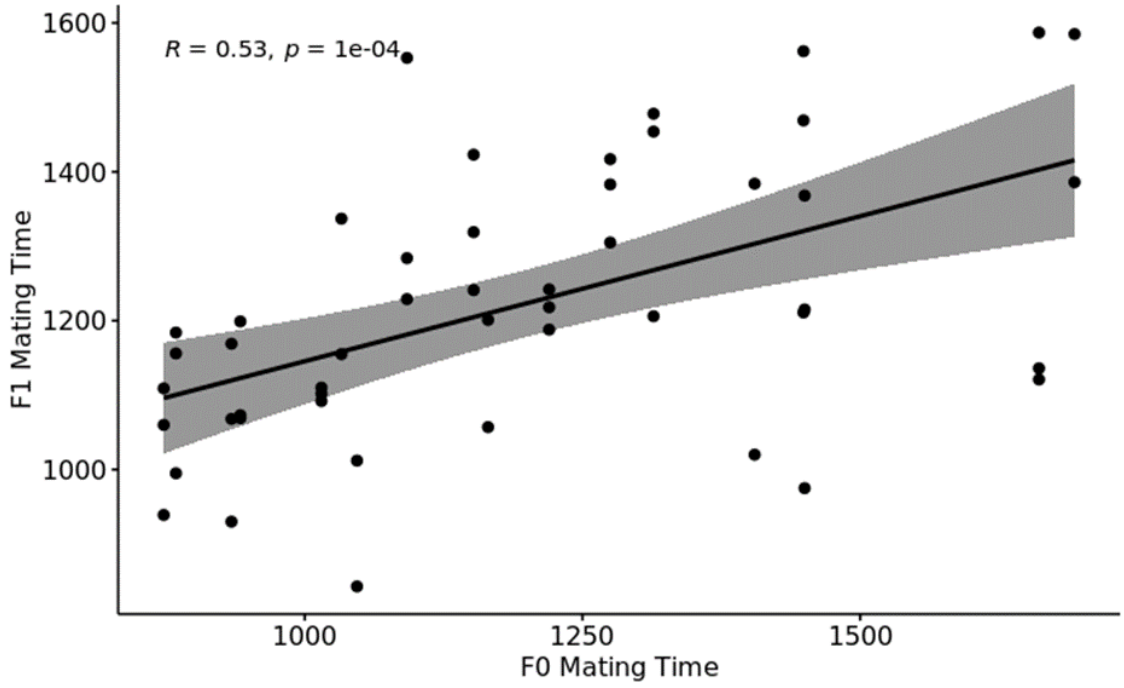


Figure 7. The correlation between the Dams (F0) mating time and the daughters (F1) mating time. Black line shows line of best fit. grey shadowing shows the accompanying 95% confidence interval.

Offspring Produced Correlation Test

There is a significant positive correlation between the number of offspring the mother produced and the number of offspring her daughters produced (R(47) = 0.32, p = 0.03; Figure 8).

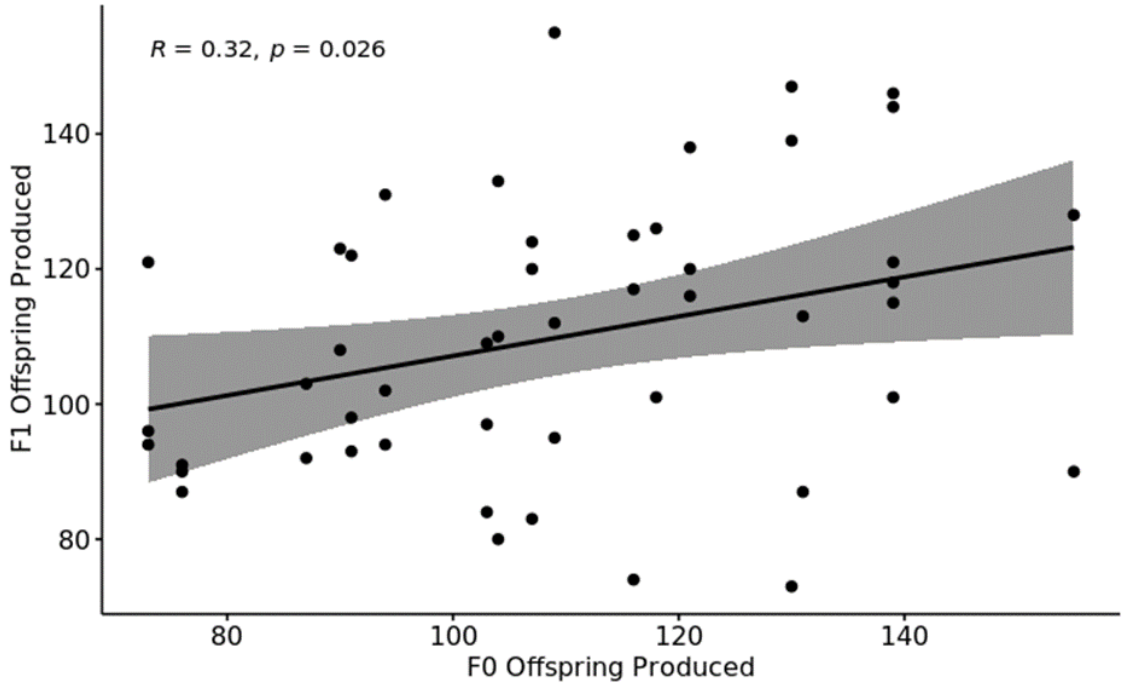


Figure 8. The correlation between the Dams (F0) offspring produced and the daughters (F1) offspring produced. Black line shows line of best fit. grey shadowing shows the accompanying 95% confidence interval.

Interactions between the correlation between mother and daughter traits and the density.

Having tested the impact, the social density treatment group had on the female flies mating behaviour, and the inherited affect the mothers had on their daughters, I created mixed model analysis to test the combined effects of mating behaviour and inheritance on mating behaviours.

Mixed Model Analysis of Courtship Latency

Daughters from mothers of low-density mothers do have a longer courtship latency, which can be explained by them inheriting the longer latency from the mothers, as flies in raised in low density environments have a longer courtship latency response to being at high density from their mothers (Table 3).

Table 2. Results of the linear (mixed) model analysis of F0 social density on F1 courtship latency.

PARAMETER	ESTIMATE	Std. Error	DenDF	F value	Pr(>F)	In final model	P-value in final model
DENSITY	92.61	94.43	8.63	0.96	0.35	1	0.109
DAM COURTSHIP LATENCY	0.63	0.21	7.56	8.79	0.02	1	0.0006
DENSITY X DAM COURTSHIP LATENCY	-0.08	0.25	7.4769	14.96	0.76	0	

Mixed Model Analysis of Mating latency

Daughters from high density mothers did not have a shorter mating latency than daughters from low density mothers (Table 4). Mothers with long mating latency are producing daughters with long mating latency, however, there is no relationship between the mother's density and the daughter's mating latency, as social density does not affect mating latency.

Table 3. Results of the linear (mixed) model analysis of F0 social density on F1 mating latency.

PARAMETER	Estimate	Std. Error	DenDF	F value	Pr(>F)	In final model	P-value in final model
DENSITY	-64.7	77.42	13.68	-0.69	0.42	1	0.19
F0 MATING LATENCY	0.27	0.10	13.68	6.61	0.02	1	0.007
Density:Dam Mating Latency	0.05	0.22	14.84	0.05	0.82	0	

Mixed Model Analysis of Mating Time

Daughters from mothers from high density social environments mate for a longer time than daughters from mothers from low density social environment (Table 5). The daughters are being directly affected by their mother's density and they are inheriting their mothers mating time, which is a response to the F0 density.

Table 4. Results of the linear (mixed) model analysis of F0 social density on F1 mating time.

PARAMETER	Estimate	Std.Er	DenDF	F value	Pr(>F)	In final model	P-value in final model
DENSITY	184.16	208.74	11	0.77	0.39	1	0.02
F0 MATING TIME	0.51	0.12	13	16.81	0.001	1	0.001
DENSITY X F0 MATING DURATION	-0.25	0.17	11	-2.19	0.16	0	

Mixed Model Analysis of Offspring Produced

Daughters from high density mothers did not produce more offspring than daughters from low density mothers (Table 6). High offspring producing mothers are producing high offspring producing daughters, however, there is no relationship between the mother's density and the daughter's offspring being produced, as social density does not affect offspring produced.

Table 5. Results of the linear (mixed) model analysis of F0 social density on F1 offspring produced.

Parameter	Estimate	Sd. Error	DenDF	F Value	Pr(>F)	In final model	P-value in final model
Density	-11.34	33.11	45	-0.11	0.73	1	0.85
F0 Offspring	0.25	0.17	45	2.12	0.17	1	0.05
Density x F0 offspring produced	0.09	0.265	45	0.31	0.75	0	

2.4 Discussion

This study set out to find out whether the social density experienced by dams have a transgenerational effect on the mating behaviour and reproductive success of their daughters. The results of this study indicate that dams can pass on transgenerational parental effects to their daughters in response to the social environment that they are reared in, with most of the of the daughters' mating traits tested in this study being affected by the social density experienced by their mother. Specifically, I found that the daughters of low-density mothers experienced a longer courtship latency and mating time but a shorter mating latency in comparison to daughters from dams that were reared under high social density. There were no effects of the number of adult offspring produced by the mother on the number of adult offspring produced by the daughter.

I predicted that the females raised at high density would anticipate increased female competition and respond by encouraging the male to initiate courtship by investing energy into producing and emitting mate attracting pheromones (Everarts et al., 2010). The results of this study support my prediction as females which were raised in a high-density treatment were courted significantly faster than females from the low-density treatment group. Although courting and the initiation of courtship is predominantly initiated and carried out by the male, there is an effect from the female's social density affecting the time it takes for the male to initiate courtship. The olfaction is an important sense in D. melanogaster courtship, which rely heavily on the use of olfactory and taste systems to detect pheromones and select their mate (Everarts et al., 2010; Borroero-Echeverry et

al., 2022), therefore the male's choice of sexual partner is dependent on the females smell and taste (Borrero-Echeverry et al., 2022; Everarts, Lacaille, and Ferveur, 2010). The results suggest that the females from the high-density treatment may "smell" more attractive males, encouraging them to initiate courtship earlier. Females in this high-density rearing environment may invest more energy into producing pheromones that attract the male fly as she is anticipating rival female competition; It is possible that the females housed in the high-density environment smelled of other females which attracted the male, however I could not find any literature to suggest that female Drosophila housed together are more attractive to males.

The courtship latency (a proxy for female attractiveness) was affected by density, but the mating latency (females' receptivity to the male) was not. The female may be reacting to the higher likelihood of a rival female by making herself more attractive to the male, but that did not mean that she was less discriminating with who she mated with or more eager to mate with them. The results of this study contrast previous studies which found that females which anticipate higher potential encounters are more discriminating when selecting mates, being less receptive to mating with the male, they have a longer mating latency (Churchill *et al*., 2021). I have not been able to find a clear explanation as to why the results in this study and previous studies contrast, but it could be caused by differences in the methods, rearing environment, or mating conditions.

Drosophila are very sensitive to the environment, which makes them an ideal model for testing hypothesis, but also means that small changes in methodology can have a large impact on the results. model for testing hypothesis, but also means that small changes in methodology can have a large impact on the results. (Allemond *et al*., 1973; Emery *et al*., 1998; Pittendrigh and Mini, 1972). It is typical for flies to be kept in 12hr light:dark cycles, however, many papers used in this study do not state their light:dark cycles, so this cannot be discounted as a reason for the differences in results. The age the flies' mate has also been demonstrated to have an impact on mating success (Giron and Casas, 2003; Ducatez *et al*., 2012; Benton, St. Claire and Plaistow, 2008), there is no standard age for mating flies, the flies in this study and Churchill (2021) study were mated at seven days old, whereas the flies in Dasgupta *et al.* (2019) were mated at two days and the Everarts *et al.* (2001) flies were mated at four days old. The differences in mating age may have contributed to the difference in results between the studies. There are other factors in the methodology that may account for the differing results, such as the time of day the flies are mated (Sakai and Ishida, 2001).

This study sought to examine the parental effects of dam's social density on the daughters' mating behaviours, and whether dams would use their own social environment to anticipate their daughters' likely social environment to pass on adaptations for their daughters to take advantage of the anticipated opportunities. The results of this study show that not only is courtship latency (used as a proxy for female attractiveness) an inherited trait with attractive mothers (dams with a low courtship latency) producing attractive daughters (progeny with low courtship latency), but the density of the mother is also influencing the courtship latency,

with daughters from low density mothers having a longer courtship latency than daughters from high density mothers. This is despite the mother's courtship latency not being influenced by their own social density, suggesting that they are passing along attractive advantages to their daughters they do not have themselves.

The results of this study also show that while the dams' mating time was not affected by their own social density, the daughter's mating time was affected by the social density experienced by their mother. Daughters from high density mothers had a longer mating duration than daughters from low density mothers, suggesting that high density mothers pass down a trait that encourages the male to mate for longer, or means the male needs to mate for longer. It is unclear what advantages a long mating time has on high density females; in males it has been suggested that male *Drosophila* use long mating times as a form of mate-guarding (Alcock, 1994), it is possible that the high density female are also using the long mating time to prevent the male from being able to find mates, or trying to ensure that through having a longer mating time she is securing more ejaculate, as she may have less mating opportunities with due to increased competition from other females. The high-density female, anticipating higher female competition, may also be using a longer mating time to ensure she receives as much ejaculate as possible, as she is anticipating fewer mating opportunities. There is not much literature to support the suggestion that longer mating time facilitates more ejaculate transfer (Edvardsson and Canal, 2006), as in many species' sperm is transferred only at the start of copulation (Duvoisin *et al*., 1999; Merritt, 1989). In *Drosophila* there is no supporting evidence that long

mating time allows for more sperm to be transferred. The results of this study contrast with previous studies which have found that mating time is inheritable in males (Gromko, 1987) but not in females (Gromko, 1989) and that females which have been housed with other females have a shorter mating time and lay fewer eggs than females kept in solitude (Churchill *et al*., 2021). I do not know if a clear reason why the results of this study contrast with other studies, other than differences in method or housing conditions of the fly. In *Drosophila*, both sexes exert control over mating time (Harai *et al*., 1999; Bretman *et al*., 2013). The experiment design of this study meant that any differences between mating time would be from female exertion, as all males were kept in the same density and rearing conditions. The density the mothers were reared in did not impact the amount of the daughter's offspring that survived to adulthood, so the advantages to longer mating could be in attributes not measured in this study, such as egg count.

Previous studies have shown that females raised in a female-biased environment produce more offspring (Moore *et al*., 2001). However, the results of our study suggest that this is not always the case, as the mother's social density did not affect how many adult offspring they or their daughters produced. There is often a trade-off between the number of offspring produced and the quality of the offspring. Although the high-density females in this study did not produce more offspring than the low-density offspring, it may be that adaptive changes were passed on to the offspring affecting qualities that were not measured in this study, such as offspring size (Allen *et al*., 2008; Reznick and Reznick, 1993; Bashey, 2006) egg size (Bonduriansky and Head, 2007) or disease resistance (Mitchelll

and Read, 2005) increasing the likelihood of survival to adulthood. Females who have been exposed to female competition will lay fewer eggs (Churchill *et al*., 2021), it was therefore predicted that the high-density females (and their daughters) would produce more offspring than the low-density offspring, however this was not the case. It could be that the high-density females lay fewer eggs, but these are of a higher quality than the low-density females' eggs (which produce more eggs). High density females have previously been shown to produce larger eggs (Bonduriansky and Head, 2007) that have better disease resilience (Mitchelll and Read, 2005), so it could be that the number of larvae that successfully hatch and reach adulthood (which is what this experiment measured) balanced between the low-density female's high quantity of eggs and offspring, and the high-density females' high quality but low quantity of eggs and offspring. However, if the environment is too competitive, they can struggle to allocate resources in maternal provisioning which leads to inferior quality progeny with reduced fitness (Christian and Lemunyan, 1958; Reznick and Reznick, 1993; Bashey, 2006). The flies in this study were kept at relatively small numbers per vial (1 or 3 vials per vial) so would never have experience malnourishment or resource competition within the experiment, which may also explain why this study found conflicting results that the social density of the parent had no adverse or positive impact on the daughter's reproductive success.

This study sought to test the effects social density, or rival competition, had on female mating behaviour and success. In order to achieve this it was important to keep female flies in large enough social densities to elicit a competitive response, without having the flies compete for resources (such as space or food

availability), therefore ruling out that any differences between females were due to differences in resource consumption or any hindrance in growth. Similar studies have on invertebrates have tended to use 7-10 female flies when assessing the effects of social density on female mating (Bonduriansky and Head, 2007; Mitchelll and Read, 2005; ; Reznick and Reznick, 1993; Bashey, 2006), however, these experiments were not testing the social environment exclusively and they did need resource provisioning and competition to be included in their experiment design (and were not using *D. melanogaster*). I decided not to include as high a number of individuals in each vial so that they would not be malnourished or compete for resources. Studies on female *D. melanogaster* (Churchill *et al.*, 2021) and male *D. melanogaster*,(Moatt, Dytham and Thom, 2013) were able to find significant effects in social densities of 3 flies per vial. Using these studies, it was decided that three female flies would be the minimum number of flies that could be kept in a vial and still trigger a competitive response. The higher density of the other studies may have influenced the quality of the offspring, possibly producing a wider variation in health due to competition for food, and there for reproductive success between the density treatments, whereas in this study all flies would have been able to have unrestricted access to resources.

Conclusion

This study set out to test whether a female can use their social environment to anticipate the mating competition their daughters are likely to experience and pass on epigenetic responses to their daughters mating behaviour which would increase their daughters' fitness in different social environments. The results of the study suggest that where the mother's mating behaviour is affected by her density (e.g., courtship latency and mating time) the daughters are also being affected by the mother's social density, with high density mothers producing daughters with shorter courtship latencies and longer mating times. This suggests that there is a parental effect being transferred to the offspring in response to the mother's social density. There is a correlation between how many offspring the mother produced and how many her daughters produced, however, neither were influenced by the density of the mother, so we cannot conclude the mother's density impacted the number of offspring produced. Overall, the results of my study suggest that there is a parental effect being passed on from mother to daughter in response to the mother's social environment, with high density mothers producing daughters which were more attractive to the male (courtship latency), and which mated for longer.

Chapter 3: Mating behaviour based on Sire Environment

3.1 Introduction

Reproduction is energetically expensive, male organisms need to invest in expensive reproductive tissue and mating behaviours in order to court successfully and produce offspring with the best chance reaching reproductive age (Nurr, 1984; Harshman and Zara, 2007; Bretman et al., 2013). It is therefore advantageous for male to be able to adapt their investment and behaviour strategies when they are exposed to resource poor environments, or when they anticipate high levels of sperm competition (when there is competition between sperm of different males to fertilise the same set of eggs). Males which experience mating competition can increase their likelihood of mating success through plasticity responses, investing in larger gametes or adapting accessory gland proteins (Linklater *et al*., 2007; Parker, 1970; McGraw *et al*., 2004), or through behavioural response, such as provisioning of nuptial gifts, which is the giving of a material gift (that is not just gametes) during sexual reproduction that improves reproductive fitness (Oberhauser, 1997; Engqvist and Sauer, 2001). For example, male dung flies, *Scatophaga stercoraria*, that were raised in high density environments where there is a higher likelihood of mating rivals are smaller than their low-density counterparts but have larger testes comparative to body size (Stockley and Seal, 2001), suggesting that the male organism can adjust their investment from developing the body to develop the testes according to the social density. Males that anticipate mating competition may also adjust their mating behaviour to reduce the risk of wasting energy investments on unsuccessful mating. *Drosophila melanogaster* males that anticipate increased male mating rivals (perceived sperm competition) will take longer to initiate

courtship, with an increased latency between mating, suggesting that they have a higher threshold in which the mating candidate must meet before they will attempt to court, limiting the amount of energy wasted on mating behaviour with a lower quality female. (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018). Fitness will be determined by intrasexual factors, such as energy invested into courtship, mating, gamete production, and female choice and receptiveness.

Drosophila species have complex courting behaviour which enables the female to observe the male anassess his quality before deciding to mate or not. The female can accept to mate with the male, delay the decision to allow her to obtain more information about competing males or reject him as unsuitable (Hoikkalla and Aspi, 1993). Male Drosophila species that are exposed to mating rivals take longer to court and mate, than those kept singularly, with an increased latency between mating (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018). I could therefore be predicted in this study that males kept in male density would be less willing to court with the female d or have longer courtship latency as they have been exposed to rival males. Males who are anticipating sperm competition will develop their plasticity responses accordingly to ensure they are able to compete.

Sperm competition is inevitable whenever rival males compete for the fertilization of a female's ova (Parker, 1970). There is a selective force towards males who can alter their provisioning or plastic responses to give themselves a competitive edge in offspring production, such as the development of larger testes (Linklater

et al., 2007), or from strategically adjusting ejaculate size in response to the current perceived sperm competition risk (Parker and Ball, 2005). Male D. melanogaster respond to high risks of sperm competition by increasing their mating time (Bretman, Fricke, and Chapman, 2009) and by increasing the number of sperm in their ejaculate (Garbaczewska, Billeter and Levine, 2013).

Investment in reproductive tissue and mating behaviour is expensive (Nurr, 1984; Harshman and Zara, 2007; Bretman *et al*., 2013). It has been demonstrated that when males are exposed to long-term nutritional stress, they restrict investment to ejaculates and testes growth (Gage and Cook, 1994). Therefore, male flies which are anticipating high levels of sperm competition may be expected to respond in a manner which would reduce their net survival cost, reducing their longevity and long-term health for investment in offspring producing plasticity traits. This is what theoretical models have predicted (Parker, 1982), however, laboratory studies on *D.melanogaster* have found significant improved survival among virgin males perceiving an elevated risk of sperm competition (Moatt, Dytham and Thom, 2013). This study will measure the longevity of male flies, to assess how the social density and mating affects their lifespan. By comparing rejected males and those allowed to mate we can begin to measure the impact mating and sperm competition have on the male's fitness. Flies exposed to sperm competition may meet costs through sacrificing resources from other functions, such as immunity defence (Simmons, 2011) or other tissues (Lamaitre *et al*., 2009). The male would need to trade-off the costs of producing offspring against their own longevity, calculating the benefits of producing offspring over a long lifespan or sacrificing their own health and longevity in favour of producing more

offspring per mating. In harsh conditions, where sperm competition is high or resources are limited, parents could gain fitness benefits from producing a few large offspring – and more numerous smaller offspring in benign environments (Fox and Mousseau, 1996; Dasgupta *et al*., 2019).

It is suggested that the high-density males may transfer ejaculates at a greater rate, becoming depleted sooner (Linklater *et al*., 2007). However, the amount of progeny the males from high sperm competition environments produce from each successive reduces at a faster rate than males from environments with lower risk of sperm competition.

The optimal between offspring size and number will depend on the relationship between offspring size and fitness, which tends to be positively correlated (Allen *et al*., 2008; Lloyd, 1987). The slope of the relationship between size and fitness depends on the environment, during adverse conditions, with higher competitive environments, it would be predicted to have a steeper offspring size/fitness relationship than, less competitive, conditions. Studies on *D. melanogaster* suggest that the social density of the father has a significant effect on the son's fitness (Dasgupta *et al.*, 2019). Suggesting that males reared at high density have an adaptive paternal effect on juvenile fitness. However, in the Dasgupta *et al.* (2019) study paternal effects on fitness were found only at the highest density (≈250 adults), which indicate that there are certain density thresholds in which certain paternal effects start affecting offspring, although this is hard to determine as the density groups were high even at the lowest density

treatment (≈70 adults) and used non-stable populations; it is difficult to determine the selection mechanism acting on the progeny fitness, whether the phenotypic difference between treatments were through paternal prediction of the offspring's fitness, or simply the individuals with better access to resources (e.g. food or egg laying space) producing fitter progeny.

This study aims to test how social density impacts the father (F0 generation) and son (F1 generation) mating behaviour, fitness, and longevity. Using stable populations and lower density treatment groups than other studies I will be able to eliminate the differences between the density groups being a result of resource competition, allowing me to test the predicted sperm competition effects the flies. I predict that the male flies in high density social environments will anticipate increased sperm competition and respond by delaying the initiation of courtship, with an increased latency between mating, as the males will be less willing to become involved in competition (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018). If the males use the social conditions in which they are raised to assess expected sperm competition; I would expect the flies raised in high density environments to have a longer mating time and produce more offspring than the low-density male flies. Again, it would be expected that their offspring would also have a longer mating time and produce more offspring comparative to those whose fathers were raised at low density. Studies have found that increased exposure to sperm competition increases the male fly's longevity, so I would predict that this study would find conquering results, however, I would also predict that the expenditure may influence the quality of offspring who as a result may have reduced lifespans (Moatt, Dytham and Thom, 2013). As it is advantageous

that the parent uses their social environment to predict the social environment the offspring are likely to encounter, it is predicted that the offspring of fathers from high density environments will also respond with the same behaviours, regardless of which density they are themselves raised.

The flies used in this study are limited to one mating, so it is therefore predicted that the flies raised in high density social environments would produce more offspring from their mating than the flies raised in low density social environments who are not expecting high levels of sperm competition. This study also wanted to look at whether these responses were passed on transgenerational to the offspring, with the male estimating the sperm competition their sons are likely to experience and provisioning accordingly.

3.2 Methods

Animal husbandry and sire density treatments

All flies used originated from a Canton-S stock population kept at 25^{o}C, on a 12-hour light:dark cycle. Stock populations were housed in 40ml plastic vials containing 7ml of an agar-based medium (40g of yeast and sucrose per litre), hereafter referred to as standard vials. They were raised in standard vials of approximately 25 *D. melanogaster*, and all vials were pooled and randomly distributed into new vials every ten days to minimise effects of inbreeding and drift. Test males were collected and sexed under light ice anaesthesia from the stock vials within nine hours of eclosion to ensure virginity and transferred to one of two treatments: solitary (one male per vial), or grouped (three males per vial), where they were kept for three days.

Females were collected within nine hours of eclosion following the last instar, before being placed into groups of three in standard vials; ensuring that they are kept virgins

Female flies were kept in groups of three flies per vial until they were three days of age. The females being from a highly inbred strain and housed in the same density groupings allow us to control for the effect of maternal environment on the fitness and courtship latency of the sire and offspring.

Mating trials

At three days old male flies were translocated to a standard vial for mating with a five-day old virgin female; the bung was forced down into the vial, leaving 2cm space above the medium for the flies to interact, increasing encounter rate and reducing time to initiate courtship. Pushing the bung down for the F1 and F0 mating also allowed me to ensure that the flies had equal space in the mating vials, so that any time difference in initiating courtship was not affected by the males taking longer to find or identify the female. It also allowed the mating vials to be standardised with the mating vials used in Chapter 2, as in both experiments the bung was pushed down into the vial, leaving 2cm above the medium for the flies to interact.

Mating behaviours were observed live, with the courtship latency (time between the flies being introduced and courtship being initiated), mating latency (time between courtship being initiated and the male successfully mounting the female) and mating time (until pair fully separates), recorded in seconds. Mating's were carried out between 08:00-11:00h GMT to minimise the time of mating having impact on the reproductive behaviours. After mating, or when they had not initiated mating for 30 minutes after introduction, the sires were removed by aspiration without anaesthesia and placed in an individual vial for the longevity assay. The standard vials containing successfully mated females were placed back in the incubator, the females were left to oviposit eggs for 24 hours before being expunged. After 21 days the emerged adults were counted, and six

virgin males taken from each vial and placed in a standard vial in either low density of a solitary male or a high density of three males per vial.

These F1 were kept in their vials for three days before being mated in the same way as their F0 fathers were mated. The mating behaviours were observed live, and courtship latency, mating latency, and mating time recorded as described above.

Longevity experiment

The longevity of the mated males was taken as a measure of mating effects on sires and to test the female antagonistic effect on male longevity. This was observed in both generations to assess whether longevity affected by density or mating with the female can be epigenetically transferred across generations. Following the mating the sire was removed and taken placed standard vial, where he was kept in isolation. Unless otherwise stated, the males were not anaesthetised, but were chilled to allow safe capture. The male's vial was kept undisturbed in an incubator at 24^{o}C, with a 12-hour light:dark cycle. Every ten days the male was aspirated into a fresh vial. Except when otherwise stated, the vials were checked daily for survival.

Statistical analysis

There were 27 F0 Flies used in this experiment, 11 high-density and 16 low-density. Once the flies that did not successfully mate was removed there were 10 high-density and 10 low-density F0 males. From these 20 F0 males the F1 generation was collected, again the flies that courted or successfully mated were removed leaving a final sample size of 107 F1 flies, 53 from high-density sires and 54 from low-density sires.

The data was analysed using R v3.6.3 (R Core Team, 2014), and the R packages “dplyr”, “ggplot2”, “tidyr” and “lme4”. The effects of density treatment on F0 mating behaviour, offspring produced, and longevity were analysed using a linear mixed effect model that used the mating behaviour as the dependent variable and the density and rearing vial as random effects. Including the vial in the model allowed me to account for the random effect of the flies rearing vial environment, as there were 4 groups of 3 flies sharing a vial.

A Shapiro-Wilk test was used to test F0 generation residuals for normality, the results showed the courtship latency did have significant departure from normality ($W = 233$, $p < 0.001$), mating latency did have significant departure from normality ($W = 0.89$, $p < 0.001$), mating time did not have significant departure from normality ($W = 0.96$, $p = 0.11$), offspring number did have significant departure from normality ($W = 0.86$, $p < 0.001$) and longevity did have significant departure from normality ($W = 0.96$, $p < 0.01$). I was unable to get the courtship latency, mating latency, offspring number and longevity normally distributed, even using

log-transformation, however I decided to use them in the mixed model anyway as it was more important to be able to include the random effects than to fulfil the assumption of normality.

A Shapiro-Wilk test was also used to test F1 generation residuals for normality, the results showed the F1 courtship latency did have significant departure from normality ($W = 0.79$, $p < 0.001$), F1 mating latency did have significant departure from normality ($W = 0.86$, $p < 0.001$), F1 mating time did have significant departure from normality ($W = 0.97$, $p = 0.03$), F1 offspring number did have significant departure from normality ($W = 0.97$, $p = 0.04$) and F1 longevity did have significant departure from normality ($W = 0.97$, $p = 0.01$). Again, using log-transformations could not get these normally distributed, however they were still used in the models as it was more important to be able to include the random effects than to fulfil the assumption of normality.

To test the test the F0 generations' social density had on their mating behaviour, longevity and offspring produced I used a linear model with the mating latency as the dependent variable and the social density, F0 vial, Sire ID and F1 vial as random effects. The F0 ID and vial was included in the model to account for the random effect of the sire, as the three sons were kept together in the high-density treatments.

I also wanted to make sure that the experiment was not impacted by being carried out over several months, so I used a linear mixed model analysis, with the mating behaviour as the dependent variable and the month the fly was mated as the independent variable. The month the flies were mated did not have a significant effect on courtship latency $F_{1,38} = 3.90$, $p = 0.06$; Appendix 1), mating latency ($F_{1,26} = 0.72$, $p = 0.40$;), mating time ($F_{1,19} = 1.12$, $p = 0.14$), or the number of offspring they produced ($F_{1,14} = 0.001$, $p = 0.97$).

3.3 Results

Not all the F0 males courted with the females in the mating vials (Low density: 77%; high density: 91%), The density of the flies did not have a significant effect on the likelihood of them initiating courtship (X^2 = 0.25 d.f. = 1, p = 0.6) or being accepted to mate with the female (X^2 = 1.66, d.f. = 1, p = 0.2).

Not all F1 male flies-initiated courtship when introduced to the female, and of the flies that did initiate courtship not all were successful in securing mating with the female. Table 6 shows the percentage of flies that initiated courted and successfully mated with the female. The F1 male's social density did not have a significant effect on whether they would court the female (X^2 = 0.54, d.f. = 1, P = 0.5) or whether the female would accept them for mating (X^2 = 0.05, d.f. = 1, P = 0.8).

Table 6. Percentage of F1 males that initiated courtship and successfully mated after initiating courtship.

			F0 (Sire) Social Density	
			Low	High
Initiated courtship	F1 (Progeny) Social Density	Low	100%	100%
		High	91%	98%
Successfully mated		Low	100%	96%
		High	98%	100%

Courtship Latency in Response to Social Density

The social density the male was housed after eclosion in had no significant effect on the courtship latency ($F_{1,3.2} = 0.05$, $p = 0.84$; Figure 9a).

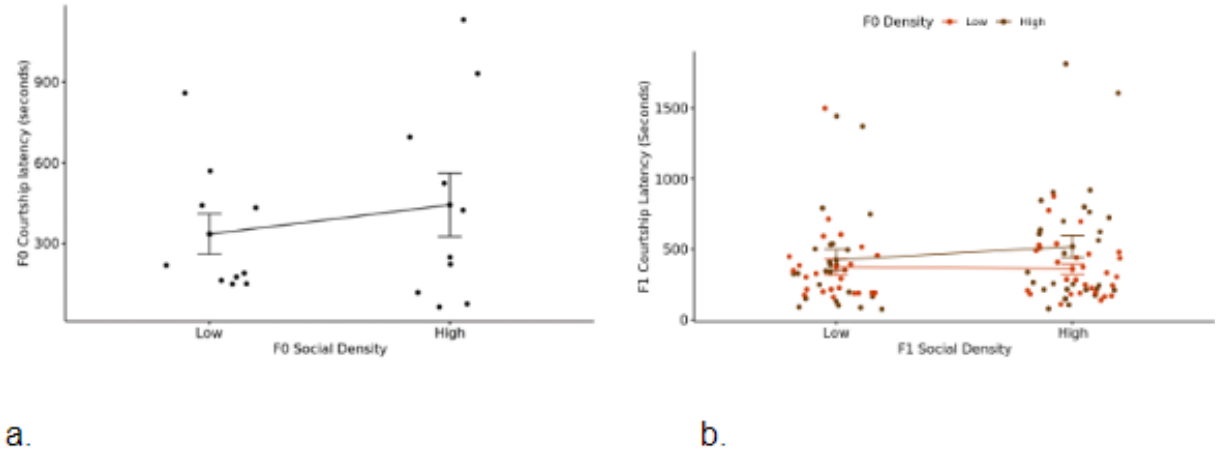


Figure 9. The effect the sire's social density had on courtship latency

F0 male flies reared in two treatment groups, low density (1 fly per vial) and high density (3 flies per vial). After mating their six of their sons were collected and put into two treatment groups, three from each male were reared in low density vials (1 fly per vial) and three from each male were reared in high density vials (3 flies per vial).

a. How long the F0 male took to begin courting after being introduced to a stock female (y-axis; in seconds) as a response to their social density (x-axis). Bar shows standard error. The social density the male was housed after eclosion in had no significant effect on the courtship latency.

b. How long the F1 male took to begin courting after being introduced to a stock female (y-axis; in seconds) as a response to their social density (x-axis). Their fathers (F0) social density is split by colour. Bar shows standard error. The social density the male was housed after eclosion in had no significant effect on the courtship latency. The sons mating latency was unaffected by neither their social density nor their fathers social density.

In the F1 generation there was no effect of the interaction between sire density and progeny density ($F_{1,93}$ = 0.29, p = 0.58). Sons from high density fathers did not take longer to initiate courtship when introduced to the female fly ($F_{1,9.3}$= 1.80, p = 0.21; Figure 9b). I also tested the interaction between the F0 social density and the F1 social density to test if there is an effect of the father's social density only for of the son's social densities. The effect of the father's social density on courting latency did not differ depending upon the son's social density (F1, 99 =1.8, p = 0.34).

F0 Male flies that were reared in high density environments did not have a shorter mating latency than male flies reared in low density environments ($F_{1,11.1} = -2.67$, $p = 0.13$; Figure 10a).

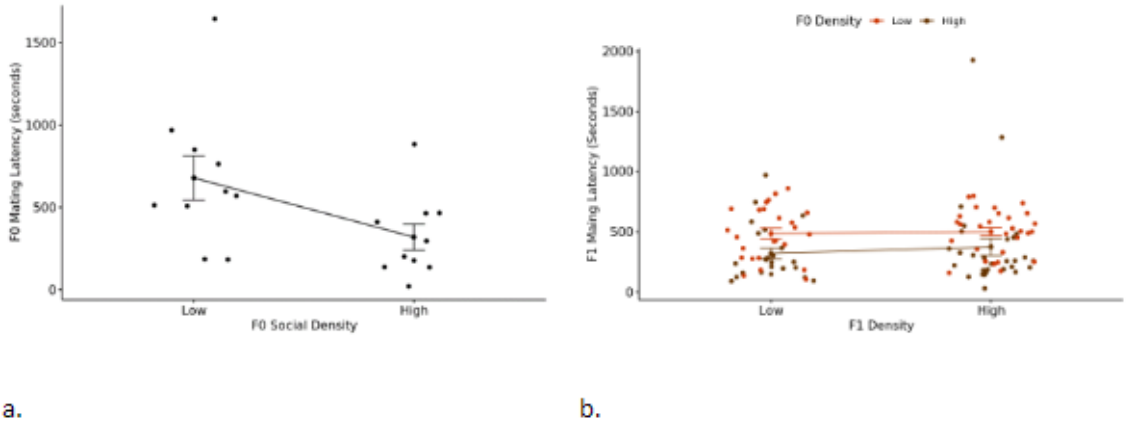


Figure 10. The effect the sires' social density had on mating latency.

F0 male flies reared in two treatment groups, low density (1 fly per vial) and high density (3 flies per vial). After mating their six of their sons were collected and put into two treatment groups, three from each male were reared in low density vials (1 fly per vial) and three from each male were reared in high density vials (3 flies per vial).

a. How long the F0 male took to spent courting before being accepted by the female (y-axis; in seconds) as a response to their social density (x-axis). Bar shows standard error. Male flies that were reared in high density environments did not have a shorter mating latency than male flies reared in low density environments.

b. How long the F1 male spent courting before being accepted by the female (y-axis; in seconds) as a response to their social density (x-axis). Their fathers (F0) social density is split by colour. Bar shows standard error. The social density the male was housed after eclosion in had no significant effect on the mating latency. The son's mating latency was unaffected by neither their social density nor their father's social density.

F1 flies from high density environments did not have a shorter mating latency than F1 flies kept at low density ($F_{1,86} = 0.39$, $p = 0.53$). Sons from high density fathers did not have a significantly longer mating latency than sons from low density fathers ($F_{1,16} = 3.03$, $p = 0.1$; Figure 10b). I also tested the interaction

between the F0 social density and the F1 social density to test if there is an effect of the father's social density only for of the son's social densities. The effect of the father's social density on mating latency did not differ depending upon the son's social density ($F_{1,81.8}$=1.14, p = 0.28).

Mating Time in Response to Social Density

Male flies from high density did not spend more time mating than male flies reared at low density ($F_{1,11} = 0.88$, $p = 0.37$; Figure 11a).

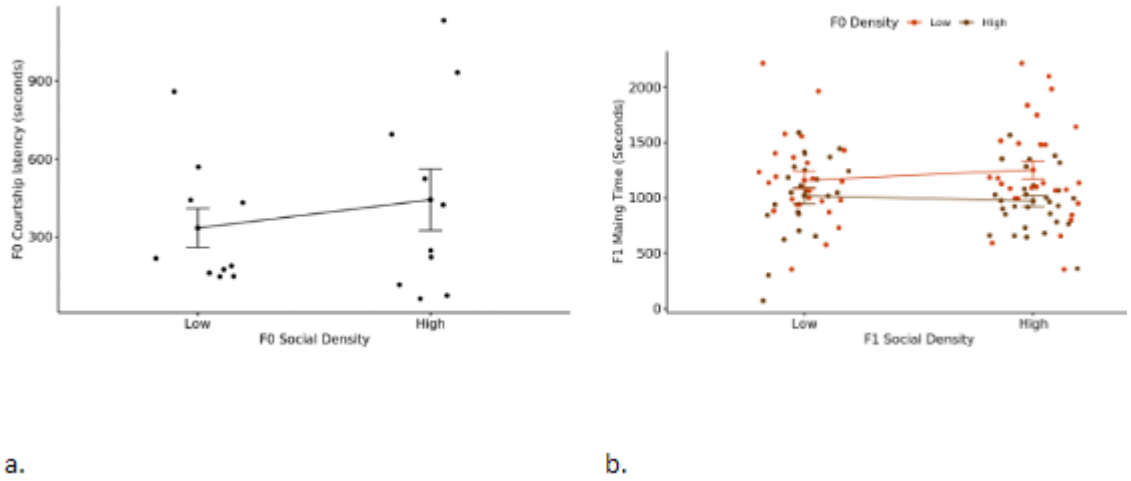


Figure 11. The effect the sires' social density had on mating time

F0 male flies reared in two treatment groups, low density (1 fly per vial) and high density (3 flies per vial). After mating their six of their sons were collected and put into two treatment groups, three from each male were reared in low density vials (1 fly per vial) and three from each male were reared in high density vials (3 flies per vial).

a. How long the F0 male took to spent attached to the female during mating (y-axis; in seconds) as a response to their social density (x-axis). Bar shows standard error. Male flies from high density did not spend more time mating than male flies reared at low density.

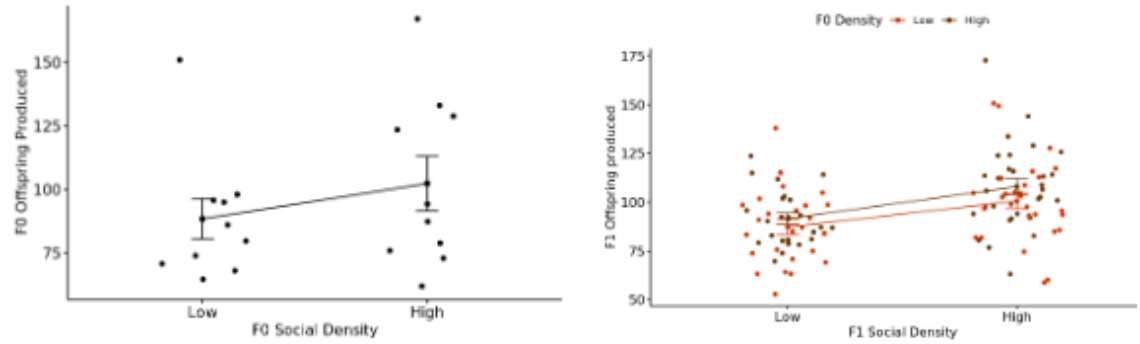
b. How long the F1 male spent attached to the female during mating(y-axis; in seconds) as a response to their social density (x-axis). Their fathers (F0) social density is split by colour. Bar shows standard error. The social density the male was housed after eclosion in had no significant effect on the mating time. The son's mating time was unaffected by neither their social density nor their father's social density.

In the F1 generation males from high density environments did not have a significantly shorter mating time than low density males ($F_{1,86} = 0.004$, $p = 0.95$). Sons (F1) from high density fathers (F0) did not spend less time mating than sons than sons from high density fathers ($F_{1,18} = 2.76$, $p = 0.11$; Figure 11b). I also tested the interaction between the F0 social density and the F1 social density to

test if there is an effect of the father's social density only for of the son's social densities. The effect of the father's social density on mating time did not differ depending upon the son's social density ($F_{1,82.7} = 1.74$, $p = 0.18$). I also tested the interaction between the F0 social density and the F1 social density to test if there is an effect of the father's social density only for of the son's social densities. The effect of the father's social density on mating time did not differ depending upon the son's social density ($F_{1,82.7} = 1.74$, $p = 0.18$).

Offspring Produced in Response to Social Density

The low-density males did not produce more adult offspring than the high-density males ($F_{1,7}$ = -1.64, p = 0.24; Figure 12a).



a. b.

Figure 12. The effect the sires' social density had on offspring produced.

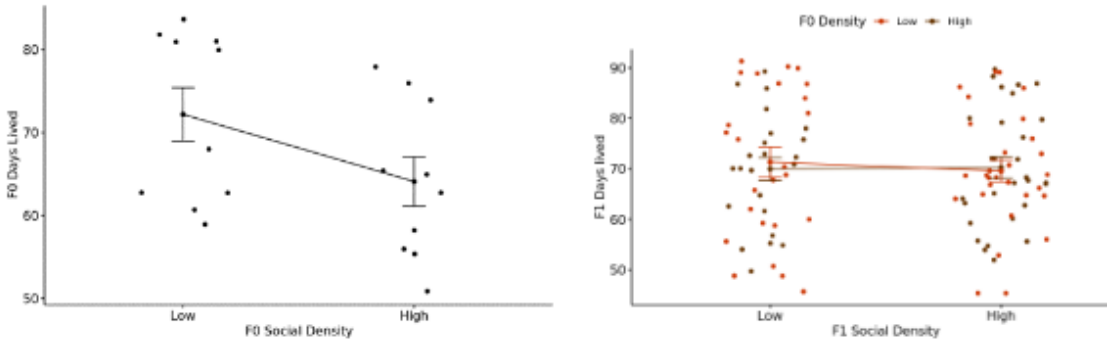
F0 male flies reared in two treatment groups, low density (1 fly per vial) and high density (3 flies per vial). After mating their six of their sons were collected and put into two treatment groups, three from each male were reared in low density vials (1 fly per vial) and three from each male were reared in high density vials (3 flies per vial).

a. The number of the F0's offspring that survived until adulthood (y-axis) following a single mating with a stock virgin female. The x-axis shows the density group of the F0 Sire. Bar shows standard error. The density the male fly was reared at did not affect the amount of adult offspring produced.

b. The number of the F1's offspring that survived until adulthood (y-axis) following a single mating with a stock virgin female. The x-axis shows the density group of the F1 Sire. The flies' fathers (F0) density treatment group is split by colour. Bar shows standard error. The number of adult offspring the F1 flies produced was not affected by either their own social density or their father's social density.

Sons from low density fathers did not produce more offspring than sons from high density fathers ($F_{1,18}$ = -0.78, p = 0.39; Figure 12b). I also tested the interaction between the F0 social density and the F1 social density to test if there is an effect of the father's social density only for of the son's social densities. The effect of the father's social density on offspring produced did not differ depending upon the son's social density ($F_{1,83.3}$ = 0.29, p = 0.77).

Male flies housed at high density did not have a reduced lifespan, with no significant difference between the longevity of high-density males and low-density males ($F_{1,10} = 2.59$, $p = 0.14$; Figure 13a).



a. b.

Figure 13. The effect the sires' social density had on longevity

F0 male flies reared in two treatment groups, low density (1 fly per vial) and high density (3 flies per vial). After mating their six of their sons were collected and put into two treatment groups, three from each male were reared in low density vials (1 fly per vial) and three from each male were reared in high density vials (3 flies per vial).

a. The number of days (y-axis) the father (F0) fly lived for following ecolosion. The x-axis shows the density treatment group of each male. Bar shows standard error. There was no significant difference between the longevity of high-density males and low-density males.

b. The number of days (y-axis) the sons (F1) lived for following their ecolosuion. The x-axis shows the density treatment group of each son, while the fathers density group is split by colour. Bar shows standard error. The sons longevity was not significantly affected by their density treatment group nor by their fathers density treatment group.

Sons (F0) from high density fathers (F1) did not have a significantly shorter lifespan than sons from low density fathers ($F_{1,19}$ = 0.007, p = 0.93; Figure 13b). I also tested the interaction between the F0 social density and the F1 social density to test if there is an effect of the father's social density only for of the son's social densities. The effect of the father's social density on longevity did not differ depending upon the son's social density ($F_{1,85.6}$ = 2.5, p = 0.11).

Correlations Between Mating Behaviours of Fathers and Sons

Courtship Latency

There was a significant positive correlation between the father's courtship latency and his son's courtship latency (R(105) = 0.45, p = <0.001; Figure 14).

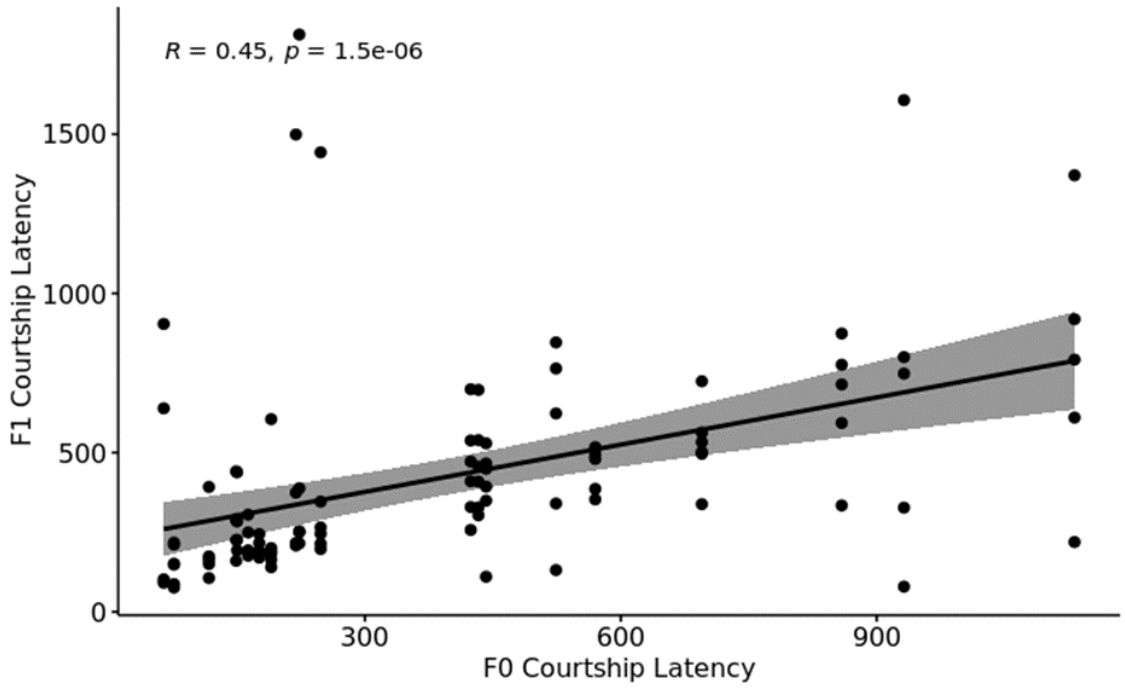


Figure 14. The correlation between the Sires(F0) courtship latency and the sons(F1) courtship latency. Black line shows line of best fit. Grey shadow shows accompanying 95% confidence interval.

Mating Latency

There was a significant positive correlation between the father's mating latency and his son's mating latency (R (105) = 0.37, p <0.001; figure 15).

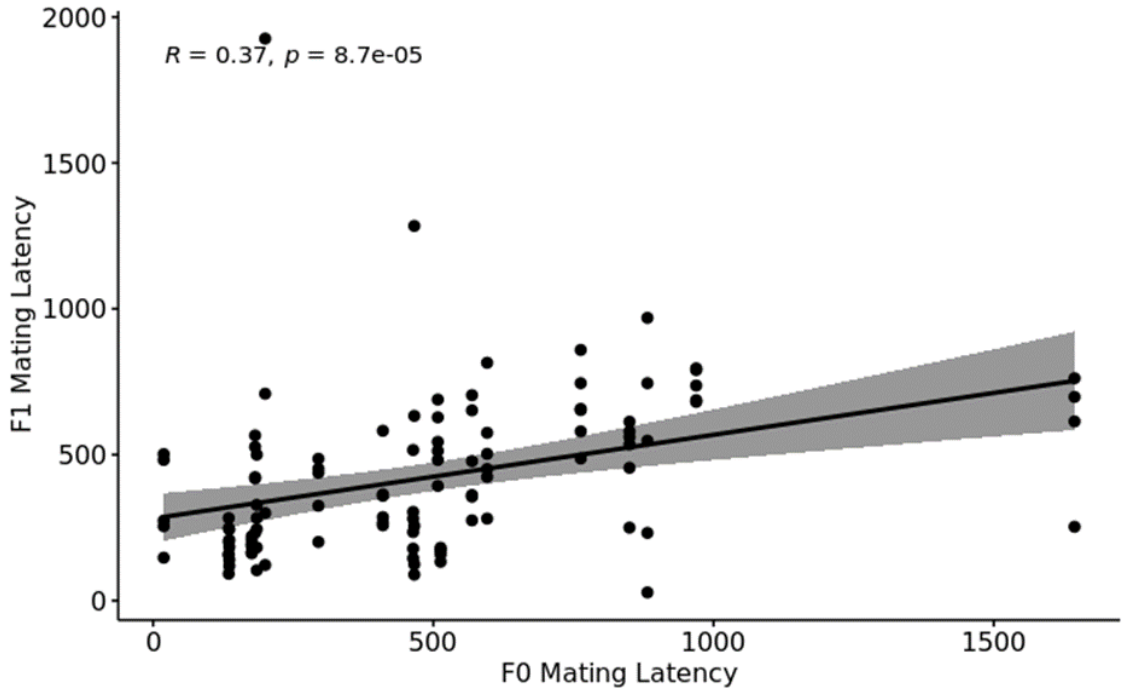


Figure 15. The correlation between the Sires(F0) mating latency and the sons(F1) mating latency. Black line shows line of best fit. Grey shadow shows accompanying 95% confidence interval.

Mating Time

There was a significant positive correlation between the father's mating time and his son's mating time (R (105) = 0.74; p <0.001, Figure 16).

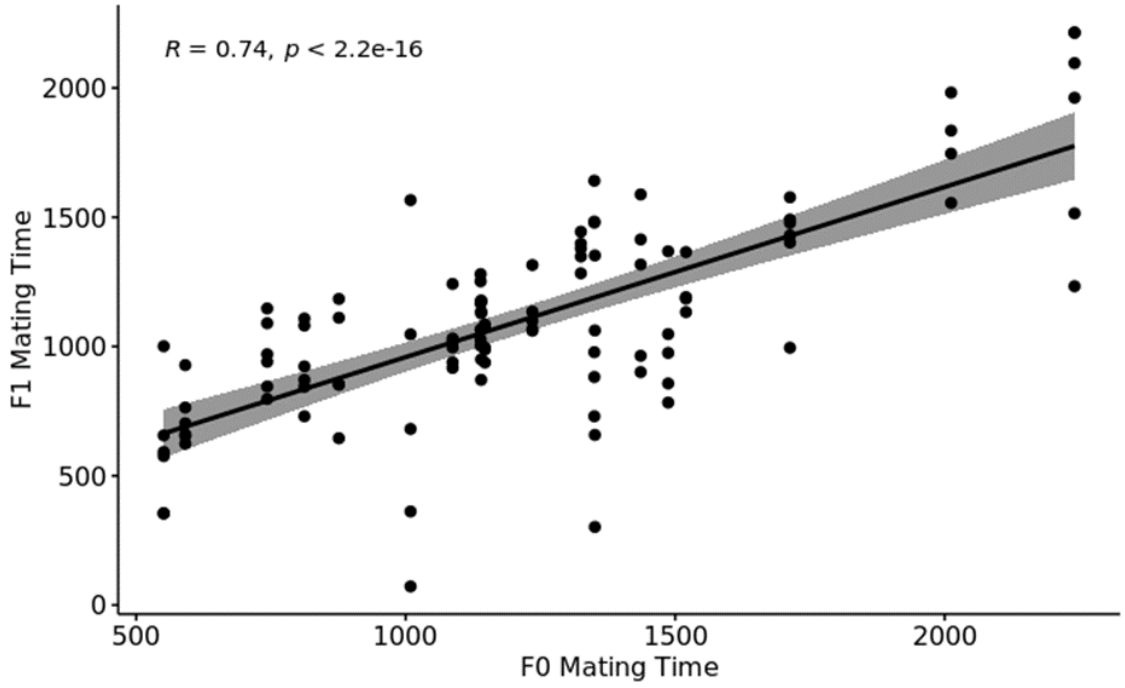


Figure 16. The correlation between the Sires(F0) mating time and the sons(F1) mating time. Black line shows line of best fit. Grey shadow shows accompanying 95% confidence interval.

Offspring Produced

There was a significant positive correlation between the number of offspring the father produced and the number of offspring his sons produced (R (105) = 0.57, p = <0.001; Figure 17).

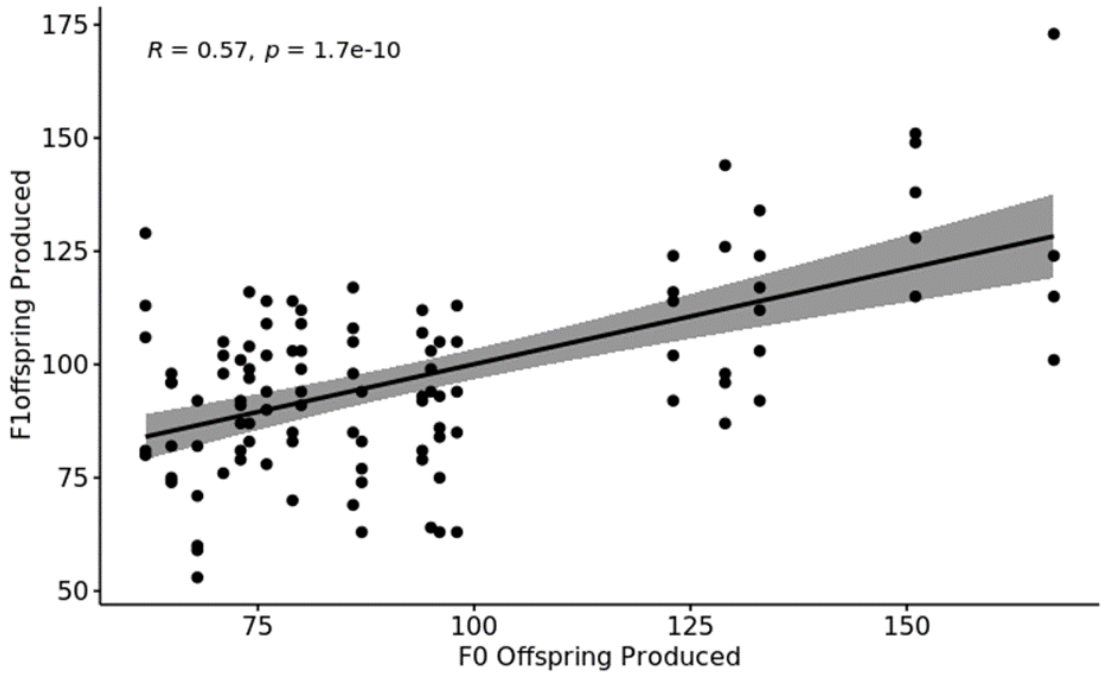


Figure 17. The correlation between the Sires(F0) offspring number and the sons(F1) offspring number. Black line shows line of best fit. Grey shadow shows accompanying 95% confidence interval.

Longevity

There was no correlation between the father's longevity and their son's longevity (R (38) = -0.02, p = 0.90; Figure 18).

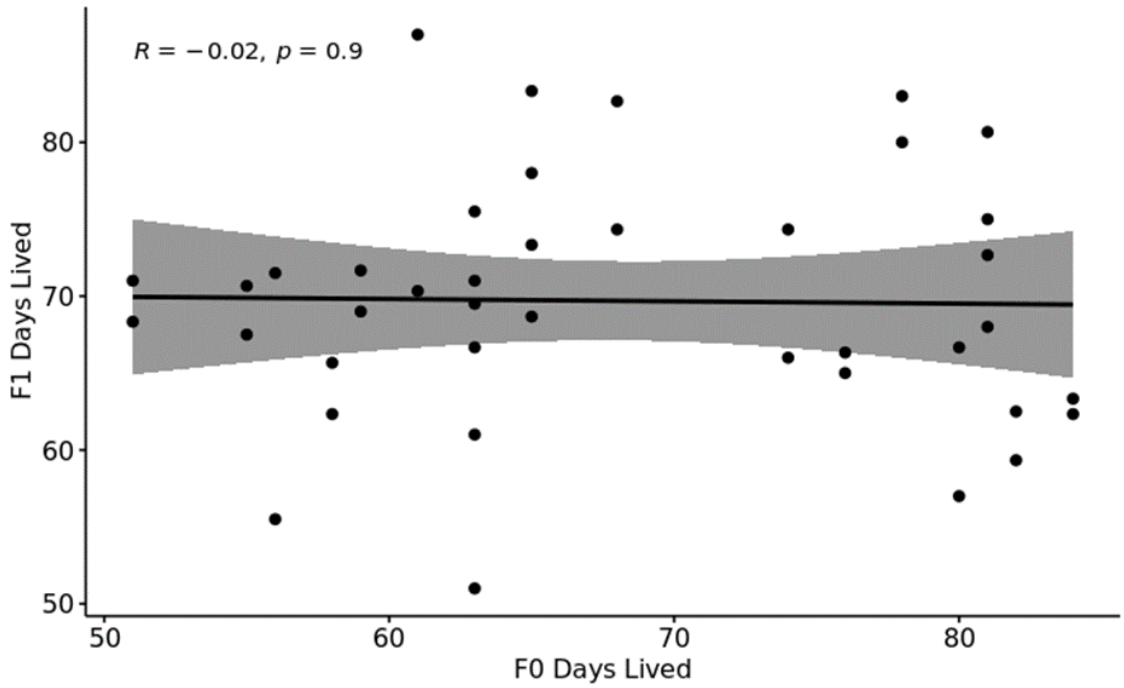


Figure 18. The correlation between the Sires(F0) longevity and the sons(F1) longevity. Black line shows line of best fit. Grey shadow shows accompanying 95% confidence interval.

Mixed Model Effects Testing Density and Inheritance Effects on Mating Behaviour

Having tested the impact the social density treatment group had on the female flies mating behaviour, and the inherited affect the mothers had on their daughters, I created mixed model analysis to test the combined effects of mating behaviour and inheritance on mating behaviours.

Mixed Model Effect on Courtship Latency

Sons from low density fathers do not have longer courtship latencies than sons from high density fathers. There is an inherited effect from the fathers but as they are not being affected by their social density, they cannot be passing down responses to their sons, if the inherited effect is removed then there is no significant effect from the father's density on the sons (Table 7; Figure 24).

Table 7. Results of the linear (mixed) model analysis of F0 social density on F1 courtship latency.

PARAMETER	ESTIMATE	Std. Error	DenDF	F value	Pr(>F)	In final model	P-value in final model
SIRE DENSITY	-212.12	134.6	99.0	16.4	0.11	1	0.31
PROGENY DENSITY	-202.33	134.1	99.0	2.5	0.13	1	0.55
SIRE COURTSHIP LATENCY	0.36	0.1	99.0	2.3	0.03	1	<0.01
SIRE DENSITY X PROGENY DENSITY	271.32	0.2	99.0	1.8	0.34	0	
SIRE DENSITY X SIRE COURTSHIP LATENCY	0.21	0.2	99.0	0.9	0.18	0	
PROGENY DENSITY X SIRE COURTSHIP LATENCY	0.26	201.5	99.0	1.3	0.25	0	
SIRE DENSITY X PROGENY DENSITY X SIRE COURTSHIP LATENCY	-0.47	0.4	99.0	0.9	0.32	0	

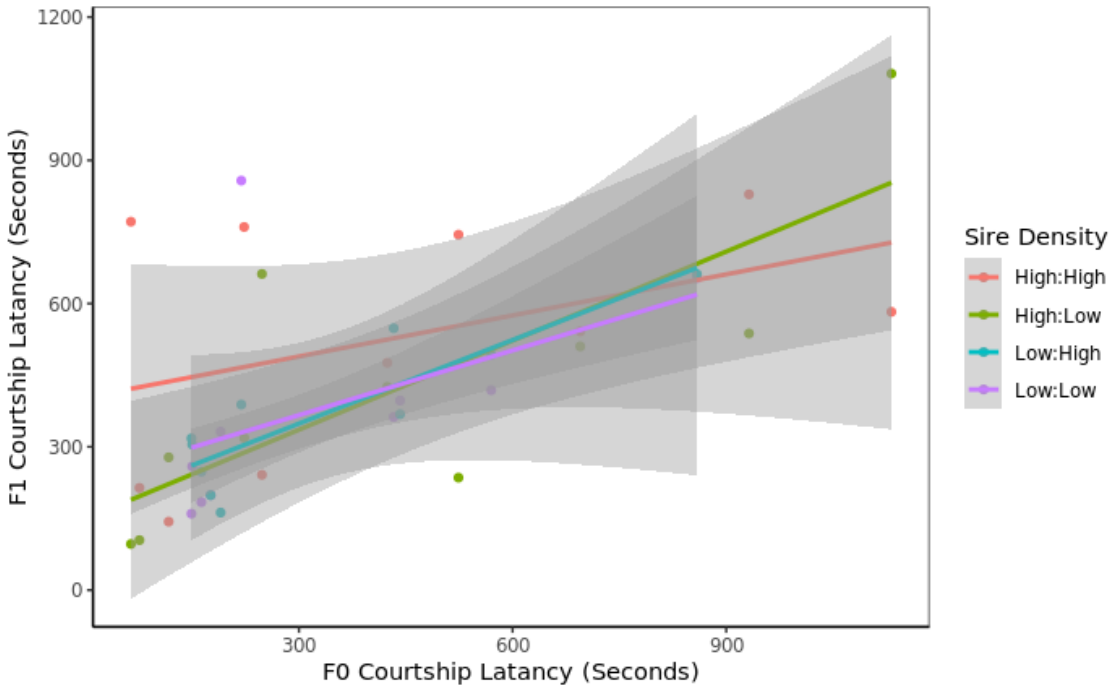


Figure 19. The relationship between the father(F0) and son (F1) density groups courtship latency. Coloured lines show line of best fit for the four treatment groups, grey shadowing shows the accompanying 95% confidence interval.

Mixed Model Effect on Mating Latency

Sons from high density fathers do not have a shorter mating latency than sons from low density fathers. The sons are inheriting their father's mating latency, but neither their mating latency nor their father's mating latency is being affected by the father's social density (Table 8; Figure 25).

Table 8. Results of the linear (mixed) model analysis of F0 social density on F1 mating latency.

PARAMETER	ESTIMATE	Std. Error	DenDF	F value	Pr(>F)	In final model	P-value in final model
SIRE DENSITY	-26.72	145.1	24.2	0.03	0.85	0	
PROGENY DENSITY	-334.63	105.4	81.1	10.08	0.002	1	0.002
SIRE MATING LATENCY	-0.16	0.2	23.70	0.27	0.60	0	
SIRE DENSITY X PROGENY DENSITY	177.02	165.5	81.8	1.14	0.28	0	
SIRE DENSITY X SIRE MATING LATENCY	0.94	0.2	80.96	11.02	0.001	1	0.001
PROGENY DENSITY X SIRE MATING LATENCY	0.32	0.2	23.6	0.95	0.33	0	
SIRE DENSITY X PROGENY DENSITY X SIRE MATING LATENCY	-0.61	0.3	81.8	4.0	0.05	1	0.04

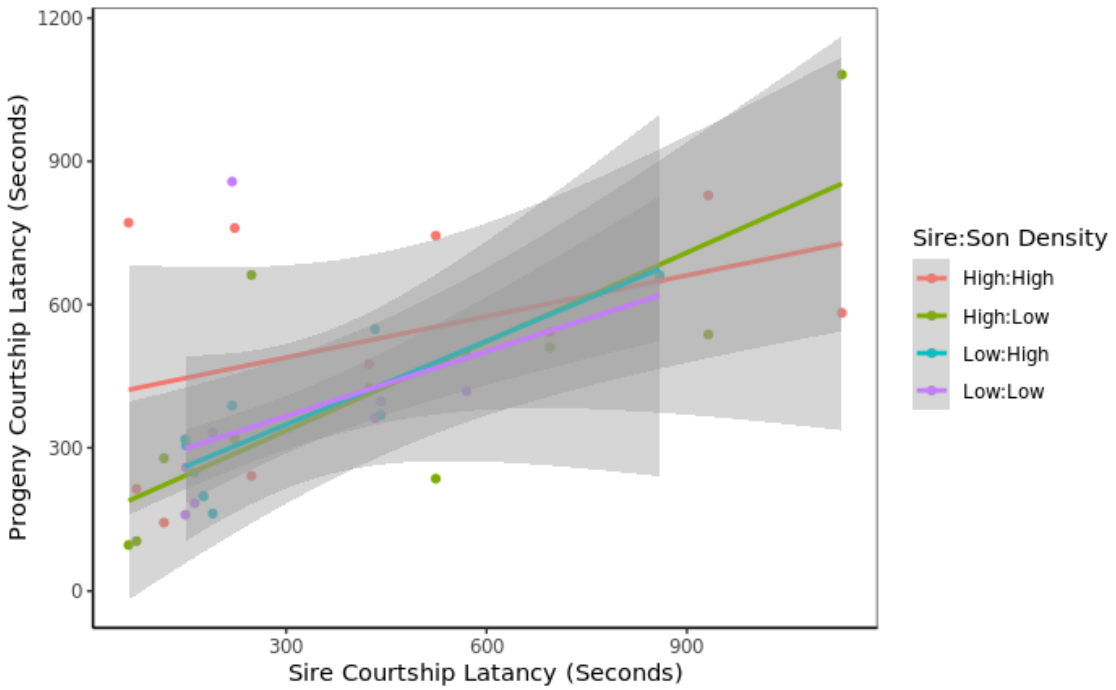


Figure 20. The relationship between the father(F0) and son (F1) density groups mating latency. Coloured lines show line of best fit for the four treatment groups, grey shadowing shows the accompanying 95% confidence interval.

Mixed Model Effect on Mating Time

Sons from high density fathers did not spend longer mating than sons from low density fathers. Sons did inherit their fathers mating time, and when this is considered in the model it shows that the son's mating time is not being affected by the father's environmental density (Table 9; figure 26).

Table 9. Results of the linear (mixed) model analysis of F0 social density on F1 mating time.

PARAMETER	ESTIMATE	Std. Error	DenDF	F value	Pr(>F)	In final model	P-value in final model
SIRE DENSITY	-435.21	261.3	32.8	2.77	0.10	1	0.11
PROGENY DENSITY	314.78	278.1	82.5	1.28	0.26	0	
SIRE MATING TIME	0.29	0.1	33.5	2.40	0.13	0	
SIRE DENSITY X PROGENY DENSITY	448.12	338.6	82.7	1.74	0.18	0	
SIRE DENSITY X SIRE TIME	0.34	0.2	82.5	1.94	0.16	0	
PROGENY DENSITY X SIRE MATING TIME	0.47	0.2	33.2	4.87	0.03	1	0.16
SIRE DENSITY X PROGENY DENSITY X SIRE MATING TIME	-0.49	0.2	83.0	3.05	0.08	1	0.08

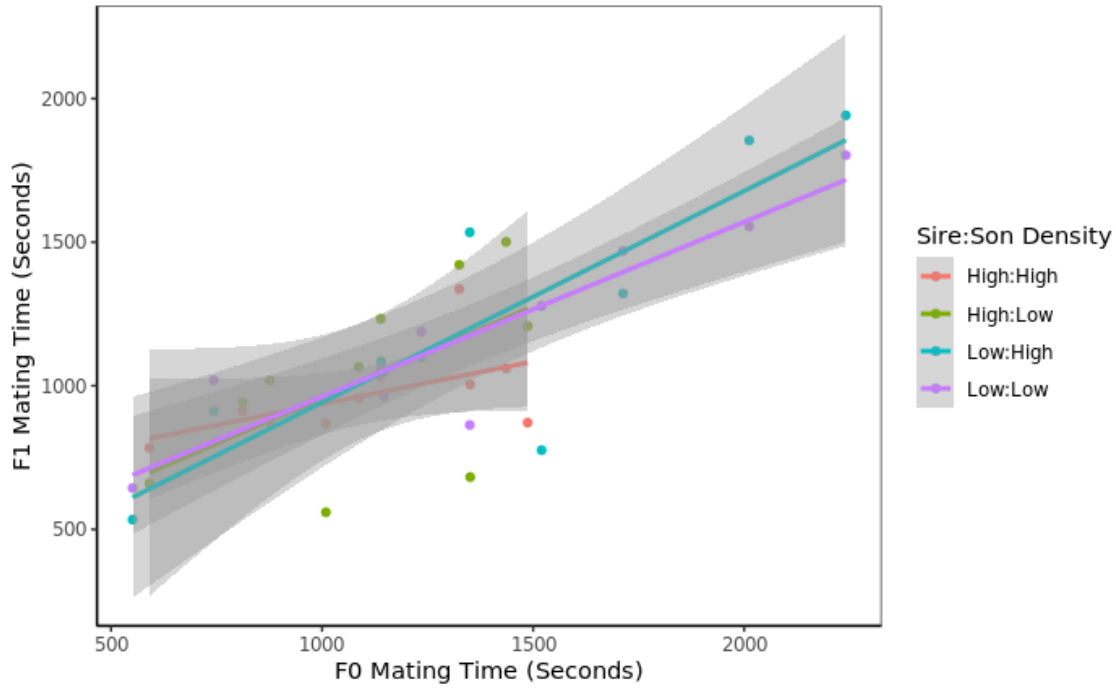


Figure 21. The relationship between the father(F0) and son (F1) density groups mating time. Coloured lines show line of best fit for the four treatment groups, grey shadowing shows the accompanying 95% confidence interval.

Mixed Model Effect on Offspring Produced

The son's number of offspring does not appear to be directly affected by their father's density, however, when the model includes the son's density and their inherited offspring production from the father it shows there is a significant effect (Table 10; Figure 27).

Table 10. Results of the linear (mixed) model analysis of F0 social density on F1 offspring produced.

PARAMETER	ESTIMATE	Std. Error	DenDF	F value	Pr(>F)	In final model
SIRE DENSITY	-18.09	18.76	26.65	0.92	0.34	1
PROGENY DENSITY	-5.24	13.04	83.48	0.16	0.68	1
SIRE OFFSPRING PRODUCED	0.40	0.11	28.98	12.14	0.001	1
SIRE DENSITY X PROGENY DENSITY	5.72	19.66	83.30	0.29	0.77	0
SIRE DENSITY X SIRE TIME	-0.11	0.12	83.46	0.86	0.35	0
PROGENY DENSITY X SIRE OFFSPRING PRODUCED	0.17	0.19	26.78	0.79	0.38	0
SIRE DENSITY X PROGENY DENSITY X SIRE OFFSPRING PRODUCED	-0.02	0.20	83.28	0.01	0.89	0

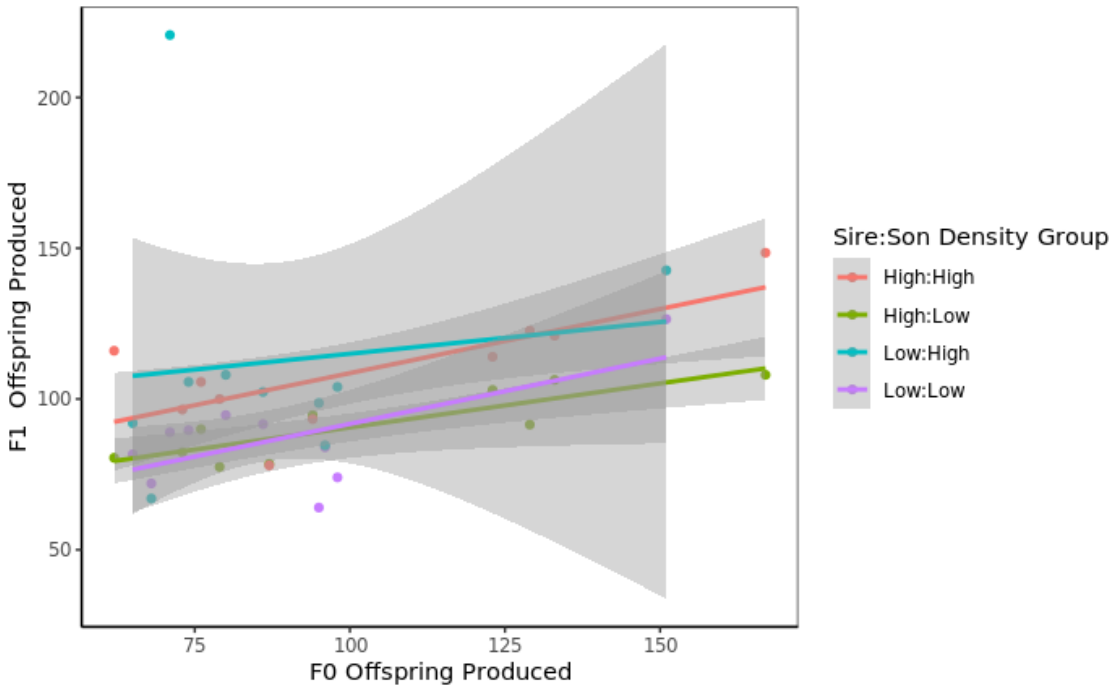


Figure 22.The relationship between the father(F0) and son (F1) density groups offspring produced. Coloured lines show line of best fit for the four treatment groups, grey shadowing shows the accompanying 95% confidence interval.

Mixed Model Effect on Longevity

Sons from high density fathers did not live longer than sons from low density fathers. Long living fathers did not produce long living sons, there is no relationship between the father's density and the life span of his offspring (Table 11: Figure 28).

Table 11. Results of the linear (mixed) model analysis of F0 social density on F1 longevity produced.

PARAMETER	ESTIMATE	Std. Error	DenDF	F value	Pr(>F)	In final model	P-value in final model
SIRE DENSITY	19.32	24.77	40.35	0.60	0.44	0	
PROGENY DENSITY	-18.07	23.68	84.18	0.58	0.44	0	
SIRE LONGEVITY	0.18	0.26	39.90	0.46	0.49	0	
SIRE DENSITY X PROGENY DENSITY	54.15	34.20	85.64	2.50	0.11	1	0.11
SIRE DENSITY X SIRE TIME	0.27	0.36	84.18	0.56	0.45	0	
PROGENY DENSITY X SIRE LONGEVITY	-0.29	0.36	9.96	0.67	0.41	0	
SIRE DENSITY X PROGENY DENSITY X SIRE LONGEVITY	-0.74	0.49	85.29	2.26	0.14	1	0.13

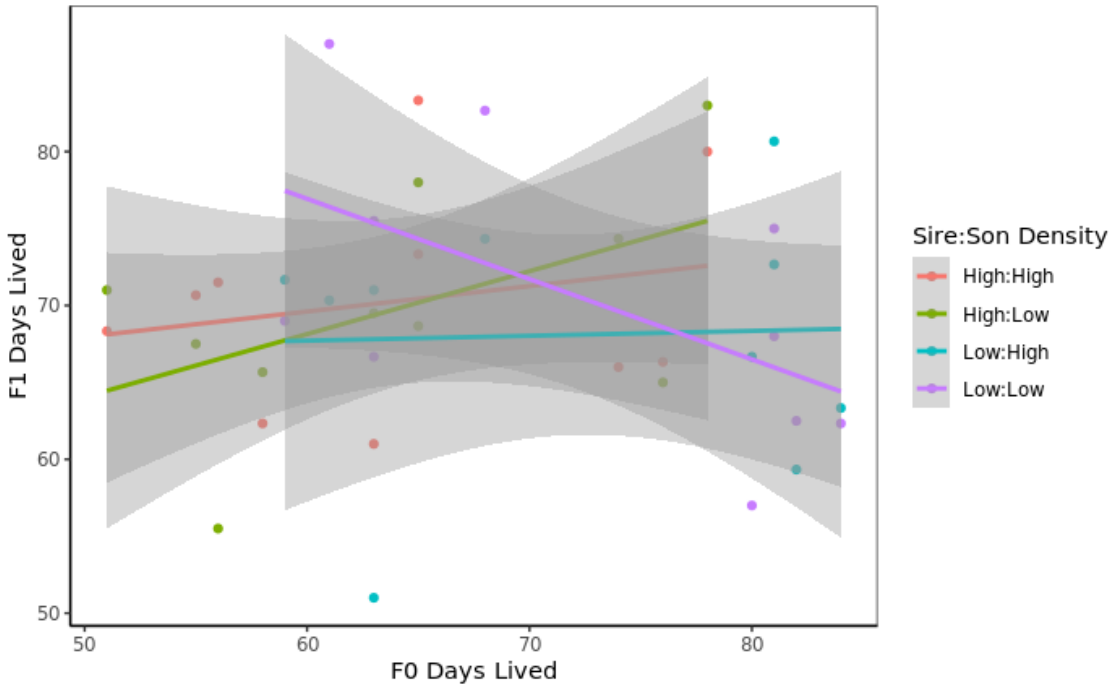


Figure 23.The relationship between the father(F0) and son (F1) density groups longevity. Coloured lines show line of best fit for the four treatment groups, grey shadowing shows the accompanying 95% confidence interval.

3.4 Discussion

This study set out to find out whether the social density experienced by sires has a transgenerational parental effect on the mating behaviour and mating success of their sons. The results of this study suggest that sires do not pass on parental effects to their sons in response to the social environment they are reared in. This study found that there was a correlation between the father and sons courtship latency, mating time and the number of offspring produced, suggesting that these mating traits could be passed down from sire to son. The sons of males at low density had a longer mating latency and longer mating times, just like their fathers, however the sons' mating traits were not affected by the father's density.

Effect of social density on sire's mating behaviour

I predicted that sires raised in high density environments would delay the initiation of courtship in response to increased anticipation of sperm competition risk, which would limit the mating opportunities for the male, meaning that the male would wish to ensure he has the best female before committing to courtship. However, unlike other studies on how social environment affects the mating behaviour of *D. melanogaster* (Bretman e*t al*., 2013; Marie *et al*., 2018), the results of this study show that males from high density social environments did not have significantly longer courtship latencies in comparison to males from low density social environments. The discrepancies between these results could have several explanations including female mate choice, anticipated sperm competition, or the males may react by provisioning energy elsewhere to ensure their energy investment is rewarded. Female choice theory suggests that a female will exhibit preferences for male traits which must be present for her to accept a mating attempt from the male (Andersson, 1994).

The high-density males may have responded to the increased sperm competition risk through other plastic responses known in *D. melanogaster*, such as strategically adjusting ejaculate size in response to the current perceived sperm competition risk (Parker and Ball, 2005; Garbaczewska, Billeter and Levine, 2013). Ejaculate size was not measured in this study, so it is possible that the flies responded to the threat of rival males through physical responses rather than through the mating behaviours measured here. It is also possible that the males tried to confer an advantage over their rivals through provisioning resources for phenotypic adaptions in their offspring such as larger testes to increase ejaculate size (Linklater *et al*., 2007), larger body size to increase attractiveness and fend off competitors (Stockley and Seale, 2005), or by investing in traits that would make them more attractive to the female, in which case we would expect the results of this study to show a shorter mating latency in flies from a high density. However, the results of this study do not support this suggestion, male flies from high density environments did not have shorter mating latency (the time between courtship and mating beginning) than males from low density environments, so we cannot conclude that they or their sons invested in traits that made them more attractive to the female.

The energy investment in courtship behaviour is expensive (Moatt, Dytham and Thom, 2013), so male flies that are not anticipating high amounts of sperm competition - such as those reared in low density environments - may try and conserve energy by not investing in attractive attributes or courtship displays, expecting the female to have a lower threshold of acceptance as there are less

males to choose from. The results of this study also showed that the mating latency, a proxy of male attractiveness, was not significantly affected by the social density the male was reared in. The mating latency is determined in part by the receptiveness of the female (Bastock and Manning, 1955; Droney, 1992; Zweerus and Groot, 2021). The female chooses whether to accept the male's courtship attempt and copulate, or reject the male (Spieth, 1952). It therefore could explain why the mating latency was not affected by the male's density, as they are largely under female control, and the females were kept in kept in the same conditions for this experiment (3 females per vial). Previous studies have shown that females which have been housed with other female more reluctant to mate they also have a longer mating latency with a shorter mating time than females kept in solitude (Churchill *et al*., 2021). This could explain why density the males were kept in did not significantly affect the mating traits of the male, as the social density of the females may have been a stronger driving force for mating latency and mating time than the male's social density. Although the acceptance or rejection of mating (and therefore mating latency) is female driven, the amount of time the fly invests in mating is male driven, and therefore the male flies could adapt their mating time investment based on the anticipated sperm competition.

Studies on how males that have been exposed to mating rivals and who are anticipating high amounts of sperm competition have generally found that males respond by increasing their mating time to allow for more sperm to be transferred (Bretman *et al*., 2009; Bretman *et al*., 2013; Enggvist and Sauer, 2001). However, this study found that exposure to rival males prior to mating had

no significant effect on mating time, with males from high density environments not having a longer or shorter mating time than males from low density environments. Males who experienced no rival competition prior to mating (low density) had lower mating success and were rejected by females more often, so there could be a selection effect where the females are already rejecting the males who they anticipate will not have the energy for an effective mating time or to produce good quality offspring. Whilst the female chooses whether to accept or reject the male, the male has choice of how energy to invest in expensive courtship behaviour, if the male does not invest enough in the courtship, the female may use this as an indicator that he has not got the energy for a long mating duration or to produce good quality or quantity offspring (Moatt, Dytham and Thom, 2013; Spieth, 1952).

That the courtship latency, mating latency and mating time were not significantly impacted by the male's social density makes sense when the density did not significantly affect the number of offspring produced. This study limited the flies to only mating once in their lifetime, it could be that if the flies were allowed to mate throughout their lives the density effects and responses to sperm competition may have been observed. Male flies who have been experienced elevated sperm competition risks will have a fitness advantage in the first few mating, but this advantage will drop off with subsequent mating (Bretman *et al*, 2013)

This study also investigated how the social density experience by the male fly would affect its longevity. Male flies which are anticipating high levels of sperm competition may be expected to respond in a manner which would reduce their net survival cost, reducing their longevity and long-term health for investment in offspring producing plasticity traits (Parker, 1970; Moatt, Dytham and Thom, 2014). Studies have shown that virgin male *D. melanogastor* exposed to high sperm competition during rearing exhibit increased survival rates (Moatt, Dytham and Thom, 2013). However, my study found that males reared in high density environments did not live significantly longer than those which were raised in low-density environments. I did find that sires that produced more offspring lived for fewer days, which would match the predicted theoretical models (Parker, 1982), as the results suggest that the male flies have provisioned their resources to increase their offspring production at the cost of their net survival. The provisioned resources would likely be physical plastic responses, such as increasing testes size (Stockley and Seale, 2001; Linklater *et al.,* 2007), and not investment in mating behaviour as neither the mating latency nor mating time had an impact on the fly's longevity.

Effect of sire's social density on son's mating behaviour

This study shows that mating behaviours, were inherited from the sire, with the sons' courtship latency, mating latency, mating time and number of offspring surviving to adulthood all being positively correlated with that of their sires. The results of this study show the social density experienced by the sire had no impact on either the sire or the son's courtship latency. However, courtship latency does appear to be inherited as courtship latency was positively correlated between

fathers and their sons. . Courtship latency is determined by several factors including how much sperm competition the male is anticipating (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018) and how attractive the female is to the male (Borrero-Echeverry et al., 2022; Everarts, Lacaille, and Ferveur, 2010). As courtship latency in this study was not affected social density, this suggests that the anticipated sperm competition did not affect males' willingness or latency to court.

I predict that the male flies in high density social environments will anticipate increased sperm competition and respond by delaying the initiation of courtship, with an increased latency between mating, as the males will be less willing to become involved in competition (Bretman *et al.,* 2013; Marie-Orleach *et al*., 2018). If the males use the social conditions in which they are raised to assess expected sperm competition; I would expect the flies raised in high density environments to have a longer mating time and produce more offspring than the low-density male flies. Again, it would be expected that their offspring would also have a longer mating time and produce more offspring comparative to those whose fathers were raised at low density. Studies have found that increased exposure to sperm competition increases the male fly's longevity, so I would predict that this study would find conquering results, however, I would also predict that the expenditure may influence the quality of offspring who as a result may have reduced lifespans (Moatt, Dytham and Thom, 2013). As it is advantageous that the parent uses their social environment to predict the social environment the offspring are likely to encounter, it is predicted that the offspring of fathers from high density environments will also respond with the same behaviours, regardless of which density they are themselves raised.

I had predicted that sons from high density fathers would have a longer mating latency than sons from low density fathers as male flies in high density social environments respond by increasing mating latency and they would pass this trait on to their sons, however contrary to this prediction there was no significant effect of the sire's social density on son's mating latency. Mating latency is controlled by the female, as she can decide to when to mate with the male, possibly delaying the decision to allow her to obtain more information about competing males, or reject him as unsuitable (Hoikkalla and Aspi, 1993). There was a positive correlation between the father's mating latency and son's mating latency, however neither was affected by the density treatment groups. The results of this study suggest that attractive sires produce attractive sons. Other studies on mating latency in D. melanogaster have found that males that anticipate increased sperm competition risk will have a longer mating latency (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018). The difference between the results of the other studies and this study could be explained in the number of males kept in each rearing vial, the male flies had a larger number of males to compete with in previous studies (N=100/7ml vial) than this study (N=3/7ml vial), so it may be that this study did not use enough males to trigger sperm competition responses in the male.

The results of this study found that the density sires were raised in prior to mating had no effect on the number of offspring sons produced. This conflicts with results from recent studies which suggest that at sufficiently high densities, males have an adaptive paternal effect on juvenile competitive fitness (Dasgupta *et al*., 2019). The experimental design of this study required relatively low number of

males in the high-density vials (N=3/7ml vial), so the male flies never had to compete for resources, with no fly being hindered in growth. This explains the discrepancy between this study and similar studies (Bretman *et al*., 2013; Marie-Orleach *et al*., 2018; Dasgupta *et al*., 2019) carried out which found that make sires kept at 'intermediate density' were themselves inferior at acquiring mates than those at high density and produced sons which were inferior at acquiring mates, as they kept their intermediate density flies in more competitive conditions (N=100/7ml vial), and therefor resource competition would have been high, as not all flies could have had equal access to resources (Dasgupta *et al.,* 2019). The higher density of the other studies may have influenced the quality of the offspring, possibly producing a wider variation in health due to competition for food, and there for reproductive success between the density treatments, whereas in this study all flies would have been able to have unrestricted access to resources.

Conclusion

This study set out to test whether a male can use their social environment to anticipate the sperm competition their sons are likely to come across and pass on epigenetic responses to their sons in terms of their mating behaviour. Overall, the results of this study suggest that whilst the sires adapted their mating behaviour in response to the social density they experienced, these responses were not passed down to their sons. Although mating behaviour was found to be inherited from the father, with positive correlations between the father and sons' courtship latency, mating time, and offspring produced, however these were not affected by the density, so we cannot conclude that the father's density has impacted these.

Chapter 4: Conclusion

The purpose of this thesis was to investigate the effect of social density on courtship behaviour in the fruit fly, Drosophila melanogaster. Specifically, it aimed to determine whether social density influences mating behaviour in male and female flies.

Based on the results of this study, it can be concluded that social density has a differential impact on courtship latency in Drosophila melanogaster, with males and females responding differently to changes in social density. Specifically, the results show that social density does not have a significant effect on courtship latency in male flies, suggesting that males may be less sensitive to changes in social density. However, the study found that social density had a significant effect on the courtship latency of female flies, with low density females being courted faster than high density females. This result suggests that female flies may be more sensitive to changes in social density and that the presence of other female flies may reduce the level of competition for male attention.

Furthermore, the study found that the social density of the parent flies did not have a significant impact on the courtship latency of their offspring, suggesting that the effects of social density on courtship behaviour may be primarily due to the immediate social environment rather than inherited traits. The finding that social density had a significant effect on the courtship latency of female flies, with low density females being courted faster than high density females, suggests that

the presence of other females may reduce the level of competition for male attention. This could have important implications for the reproductive success of female fruit flies in the wild. For example, in high-density populations, where competition for mates may be more intense, female fruit flies may be at a disadvantage if they are not able to court and mate quickly enough. By contrast, in low-density populations, where competition for mates is likely to be lower, female fruit flies may have a greater chance of attracting a mate and reproducing successfully. Moreover, the finding that social density did not have a significant effect on courtship latency in male flies suggests that males may be less sensitive to changes in social density. This could have implications for the evolution of courtship behaviour in fruit flies, with males potentially evolving to be less responsive to social cues in high-density populations where competition for mates is more intense.

Knowing that males tend to court low density females more quickly than high density females, could potentially have implications for conservation efforts, particularly in managing populations of endangered or threatened species. Understanding the factors that influence courtship behaviour can help conservationists to develop strategies that promote successful reproduction in these populations. For instance, if low population density is associated with increased courtship activity, then efforts to reduce population density (e.g., through habitat restoration or predator control) may help to stimulate courtship and increase breeding success.

Furthermore, understanding the factors that influence courtship behaviour can also help conservationists to identify and protect critical habitat area. For instance, if the presence of specific plants or other resources influences courtship behaviour, then these resources can be targeted for protection and restoration.

The results of this study suggest that there is no significant difference in the mating time of male flies from high-density environments compared to those from low-density environments. Similarly, there is no significant difference in the mating time of female flies from low-density environments compared to those from high-density environments. However, there is a significant difference in the mating time of daughters from low-density mothers compared to those from high-density mothers. Sons from high-density fathers did not show a significant difference in mating time compared to those from low-density fathers.

These results matter to wild *D. melanogaster* because they shed light on the impact of social density on mating behaviour. Previous studies have shown that social density can have a significant effect on the mating behaviour of *D. melanogaster*. However, this study suggests that the effect of social density on mating behaviour may be more complex than previously thought. The finding that daughters from low-density mothers had a shorter mating time than those from high-density mothers suggests that the social environment of the mother may have a significant impact on the mating behaviour of their offspring. One possible explanation for this sex-specific effect is that the eggs produced by female flies are larger and more nutrient-rich than the sperm produced by male flies. This

means that the maternal environment can have a greater impact on the development and behaviour of offspring than the paternal environment. In Drosophila melanogaster, the eggs are fertilized internally by the sperm, and the developing offspring are nourished by the mother's yolk before hatching. Thus, the maternal environment can have a significant impact on the development of offspring, including their behaviour. It is possible that the differences in the maternal environment between high-density and low-density environments had a greater impact on the development of the daughters than the sons, leading to differences in their mating behaviour. On the other hand, the finding that sons from high-density fathers did not spend less time mating than sons from low-density fathers suggests that the paternal environment may not have as strong an impact on the mating behaviour of offspring. This could be because the sperm produced by male flies contain less nutrient content than the eggs produced by female flies. Therefore, the paternal environment may not have as much influence on the development and behaviour of offspring as the maternal environment. The sex-specific effect of social environment on mating behaviour observed in this study could be because the maternal environment has a greater impact on the development and behaviour of offspring than the paternal environment in Drosophila melanogaster. Therefore, daughters may be more sensitive to differences in the maternal environment than sons, leading to differences in their mating behaviour.

In conclusion, this thesis has explored the impact of social density on courtship behaviour in Drosophila melanogaster. The findings of this study demonstrate that social density has a differential effect on courtship behaviour in male and

female flies. Male flies appear to be less sensitive to changes in social density, while female flies are more sensitive to social cues and compete for male attention. Additionally, the study found that the social environment of the mother has a greater impact on the mating behaviour of offspring than the paternal environment. These findings have important implications for understanding the factors that influence courtship behaviour in fruit flies and could potentially inform conservation efforts aimed at promoting successful reproduction in endangered or threatened populations. Future studies could investigate the underlying mechanisms that drive these sex-specific effects and explore whether they are driven by differences in the perception of social cues or differences in the hormonal or neural mechanisms that regulate courtship behavior. Overall, this study highlights the importance of considering social context when studying courtship behaviour in Drosophila melanogaster and other species.

Appendix 1. The Effect the Month had on Mating Behaviour

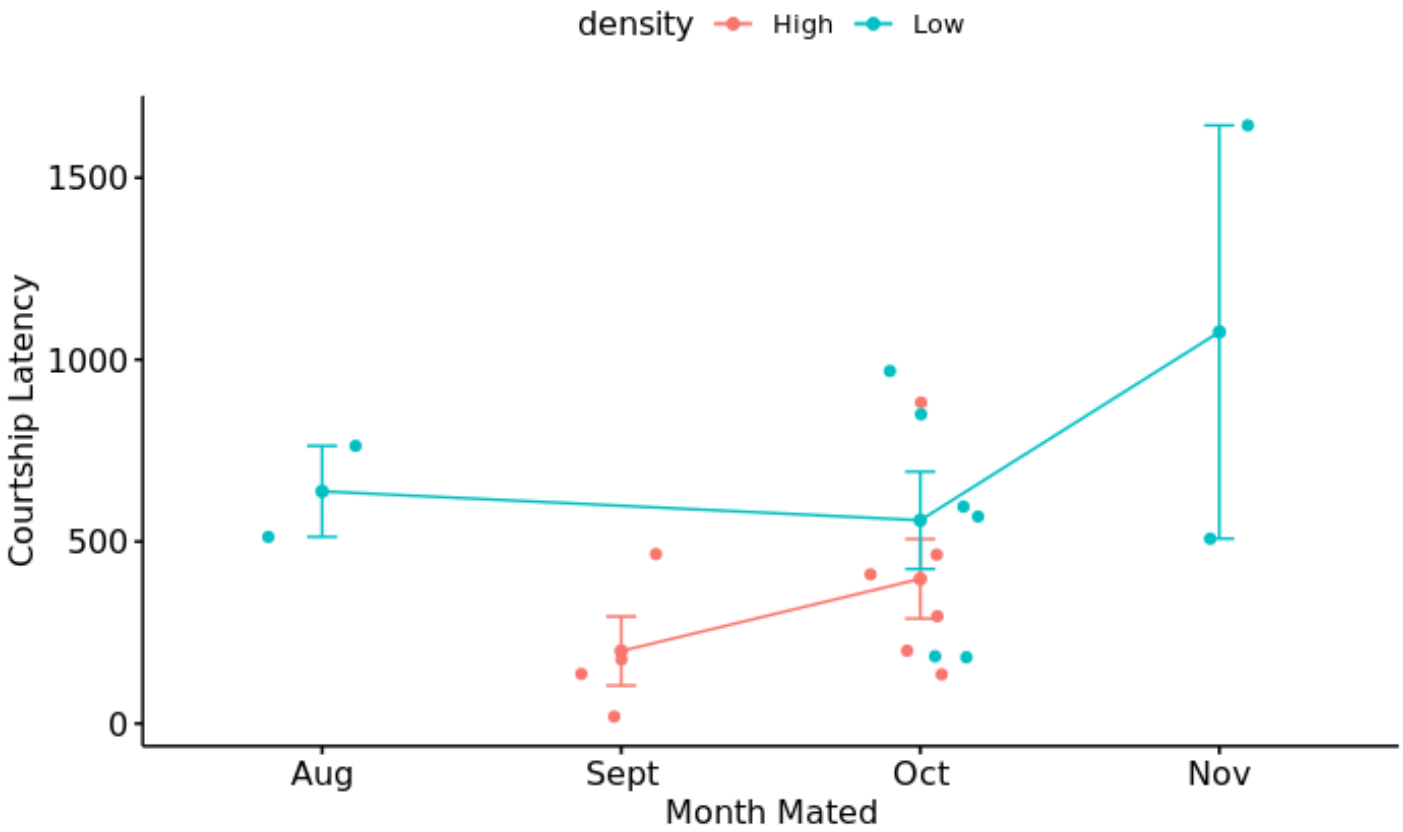


Figure 24. The effect the month the F0 sires were mated had on latency to be begin courting. Means (black dot) and 95% confidence intervals of courtship latency are shown in seconds. Red line shows line of best fit for low density males, blue line shows line of best fit for high density males

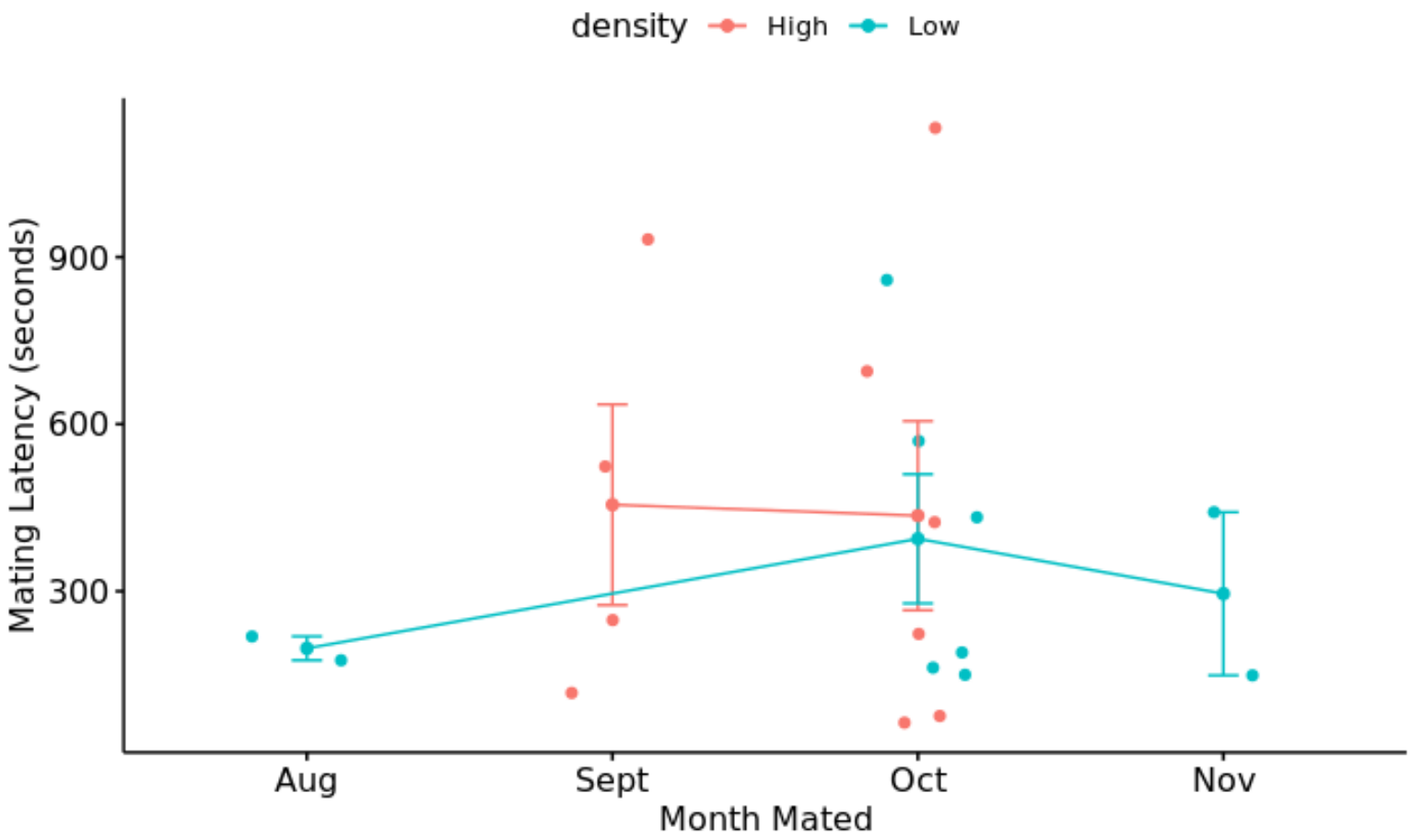


Figure 25.The effect the month the F0 sires were mated had on mating latency. Means (black dot) and 95% confidence intervals of courtship latency are shown in seconds. Red line shows line of best fit for low density males, blue line shows line of best fit for high density males

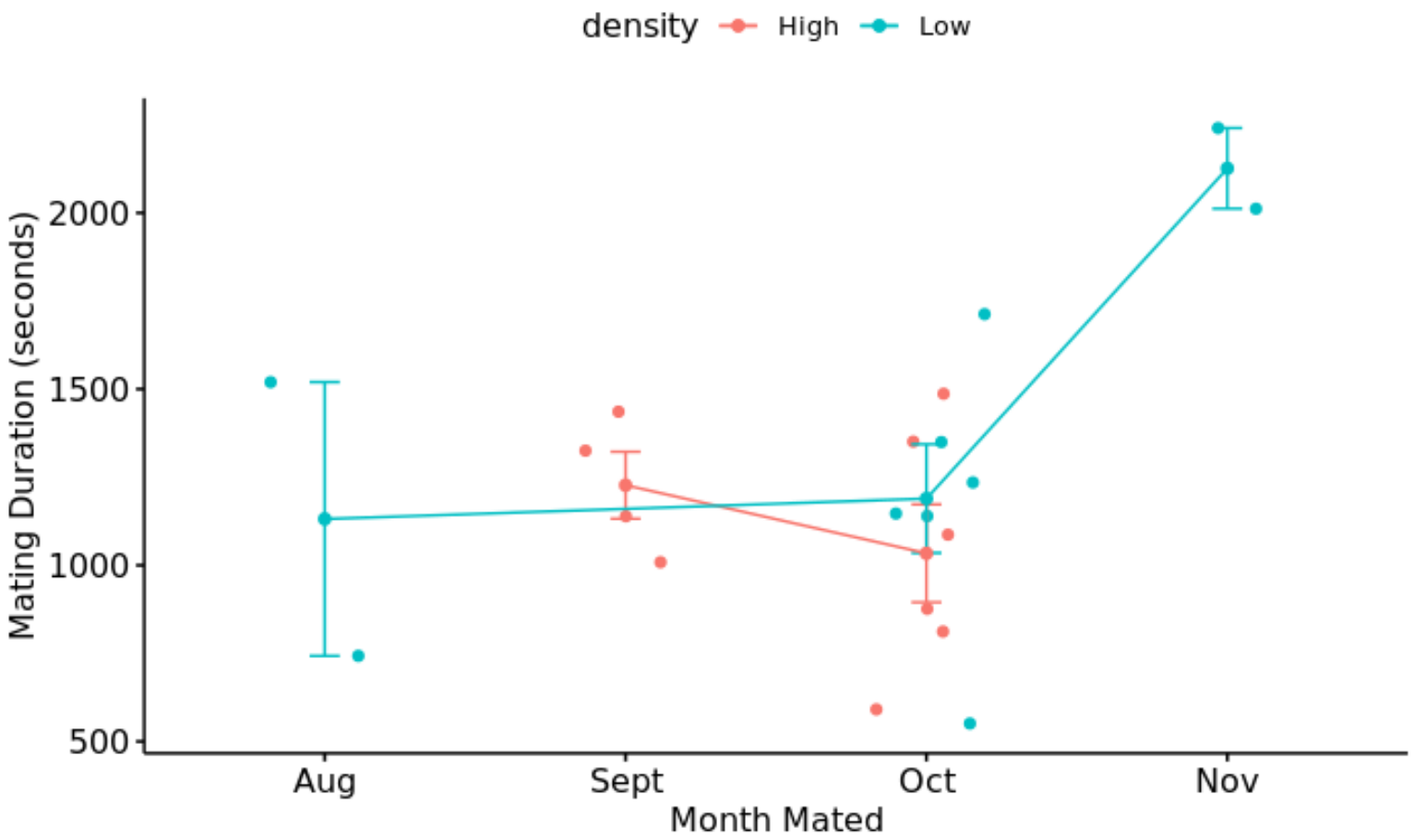


Figure 26. The effect the month the F0 sires were mated had on mating time. Means (black dot) and 95% confidence intervals of mating time are shown in seconds. Red line shows line of best fit for low density males, blue line shows line of best fit for high density males

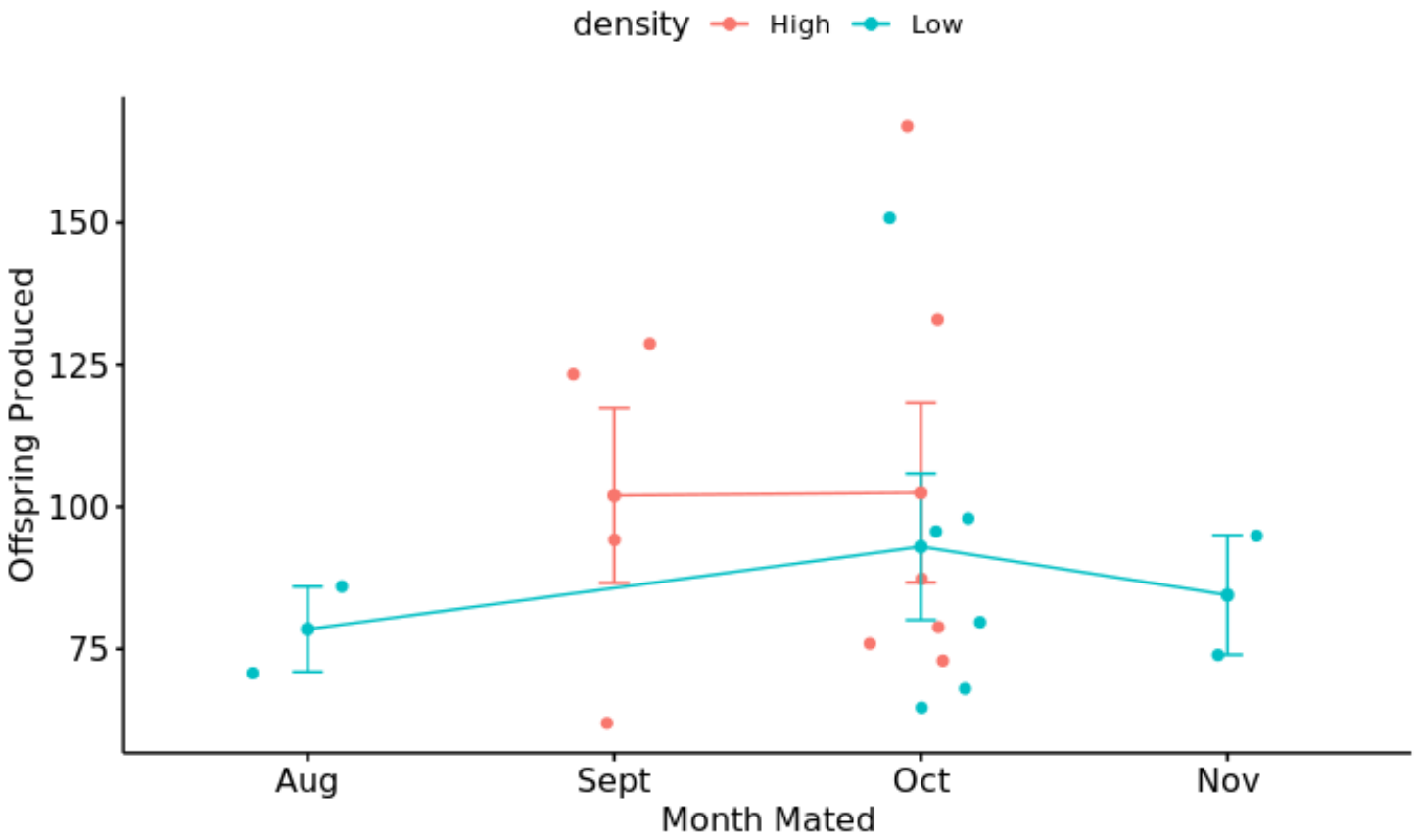


Figure 27. The effect the month the F0 sires were mated had on the offspring produced from the mating. Means (black dot) and 95% confidence intervals of offspring produced. Red line shows line of best fit for low density males, blue line shows line of best fit for high density males

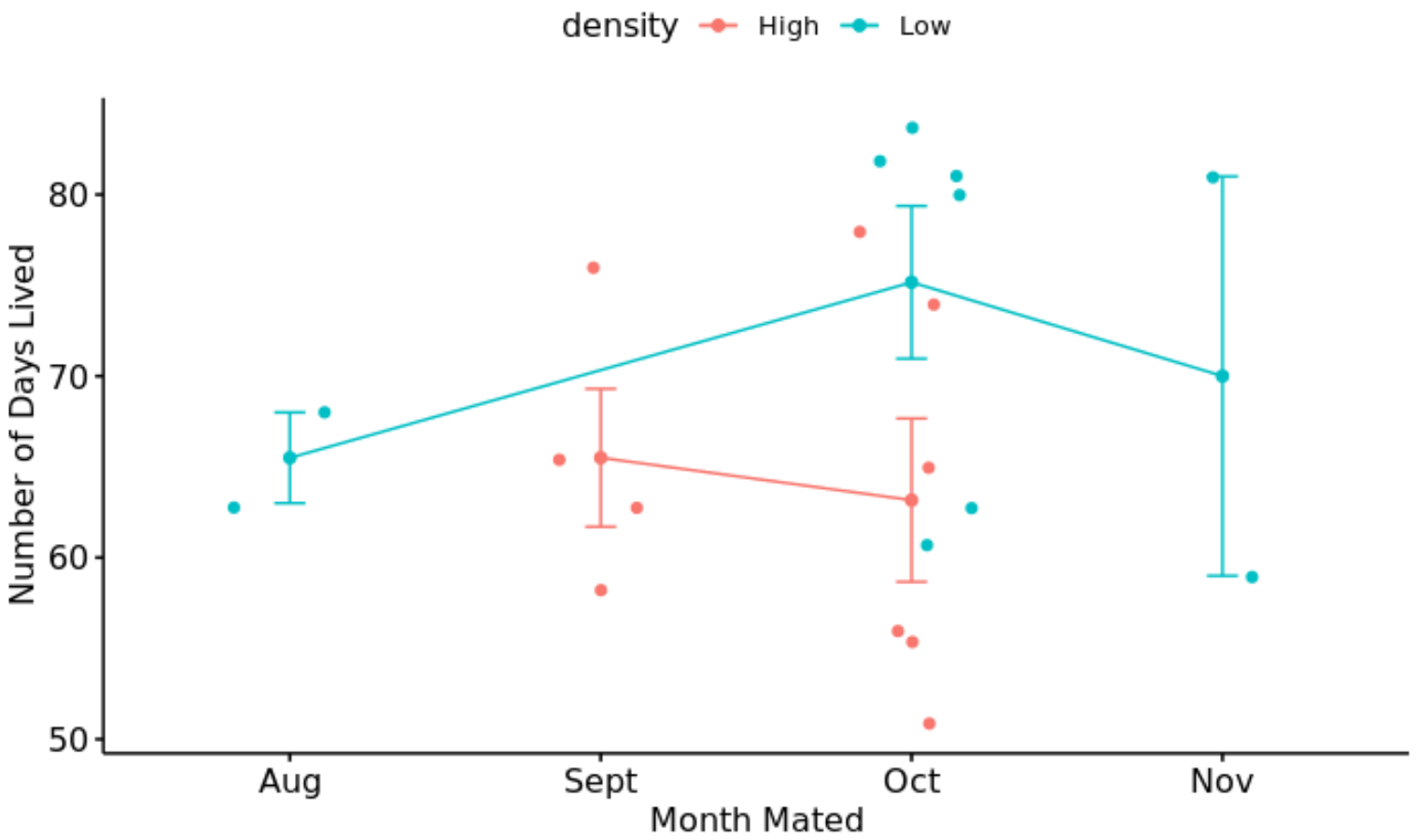


Figure 28. The effect the month the F0 sires were mated had on the longevity (number of days lived). Means (black dot) and 95% confidence intervals of offspring produced. Red line shows line of best fit for low density males, blue line shows line of best fit for high density males

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